The detection, outcome and molecular biology of pre-invasive lesions of the bronchus.

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Declaration

I Anindo Kumar Banerjee confirm that the work presented in this thesis is my own. Where information has been derived form other sources, I confirm that this has been indicated in the thesis.

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Abstract of thesis

Introduction: It is proposed that squamous cell carcinoma of the bronchus develops from carcinogen-exposed epithelium through a series of pre-invasive lesions of increasing histological and cytological abnormality. This has not been reliably demonstrated, and it is not known whether pre-invasive lesion development follows a predictable time-course and pattern or whether all pre-invasive lesions are committed to the development of malignancy. Pre-invasive lesions manifest genetic changes similar in pattern to that of squamous cell carcinoma. The accumulation of genetic damage, as a consequence of prolonged carcinogen exposure, may drive the progression of an individual pre-invasive lesion to malignancy, and the ultimate pattern of genetic changes may determine the outcome of that lesion.

Methods: In the present work patients with pre-invasive lesions underwent serial bronchoscopy and biopsy to determine the natural history of pre-invasive lesions. Serial biopsies from lesions under follow-up were studied histologically and using loss of heterozygosity analysis at chromosomal loci thought to be involved in the pathogenesis of squamous cell carcinoma.

Results: The natural history of pre-invasive lesions is variable. Some lesions progress, some regress and some remain unchanged histologically. Different lesions in a single patient may have different natural histories and different outcomes. Short-term follow-up may misrepresent the long-term evolution of an individual lesion or bronchoscopic location. Molecular studies showed that different lesions in individual patients appeared to have originated from a single progenitor cell, but acquired significant genetic differences during lesion development. Progression of pre-invasive lesions to carcinoma was associated with loss of heterozygosity along the majority of 3p with loss at 9p and the acquisition of 4p16 loss at the transition from carcinoma-in-situ to invasive disease. Regression to normal epithelium was associated with the failure to acquire these changes at the same time.

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Part 1: Introduction

Lung cancer is the commonest cause of death due to malignant disease in the United Kingdom but despite advances in imaging and treatment the prognosis of lung cancer sufferers has not changed significantly in the past 40 years[1]. The cure rate remains at 7-13%, due in part to the late stage at which the disease presents in the majority of patients[2]. Evidence that the treatment of early stage and radiologically occult lung cancer improves long-term survival has stimulated research efforts into methods of early detection and screening[3,4]. Studies using spiral computed tomography and sputum analysis have shown some promise[5,6]. However, only established invasive carcinomas are detected using these modalities, a stage at which metastatic spread may already have occurred, and currently their effect on long-term survival is not known. Interest has therefore turned to even earlier stages of lung cancer development, when the disease has not yet become frankly invasive.

Pre-invasive lesions of the bronchus

Pre-invasive lesions are defined as "a precursor lesion of squamous cell carcinoma arising in the bronchial epithelium. Squamous dysplasia and carcinoma-in-situ are a continuum of recognisable histologic changes in the large airways. They can occur as single or multifocal lesions throughout the tracheobronchial tree. Dysplasia or carcinoma-in-situ may exist as an isolated finding or as a bronchial surface lesion accompanying invasive carcinoma"[7] (figure 1.1). The basement membrane is intact in pre-invasive lesions[8], and there is no possibility of metastatic spread, which is in contrast to squamous cell carcinoma where there is the potential for metastasis as soon as invasion occurs. There is some evidence that a 90% 5-year survival might be achieved by treating lesions at the pre-invasive stage[3,9]. Consequently, pre-invasive lesions are thought good candidates for therapy, with the aspiration that their treatment will improve long-term survival from carcinoma of the bronchus. Large studies have shown that only 10% of heavy smokers develop carcinoma of the bronchus[10,11]. The studies of Auerbach showed that up to 75% of heavy smokers and patients with previous lung cancers may harbour pre-invasive lesions of the bronchus[12], which suggests that although pre-invasive lesions are prevalent within the bronchial tree of smokers, not all pre-invasive lesions are committed to progress to carcinoma. This raises questions regarding their treatment, as lesions that are not at risk of malignancy should not

require intervention. To identify which lesions are at risk and target treatment appropriately, and to interpret the results of studies of treatment of pre-invasive lesions, a detailed understanding of the natural history of pre-invasive lesions is necessary.

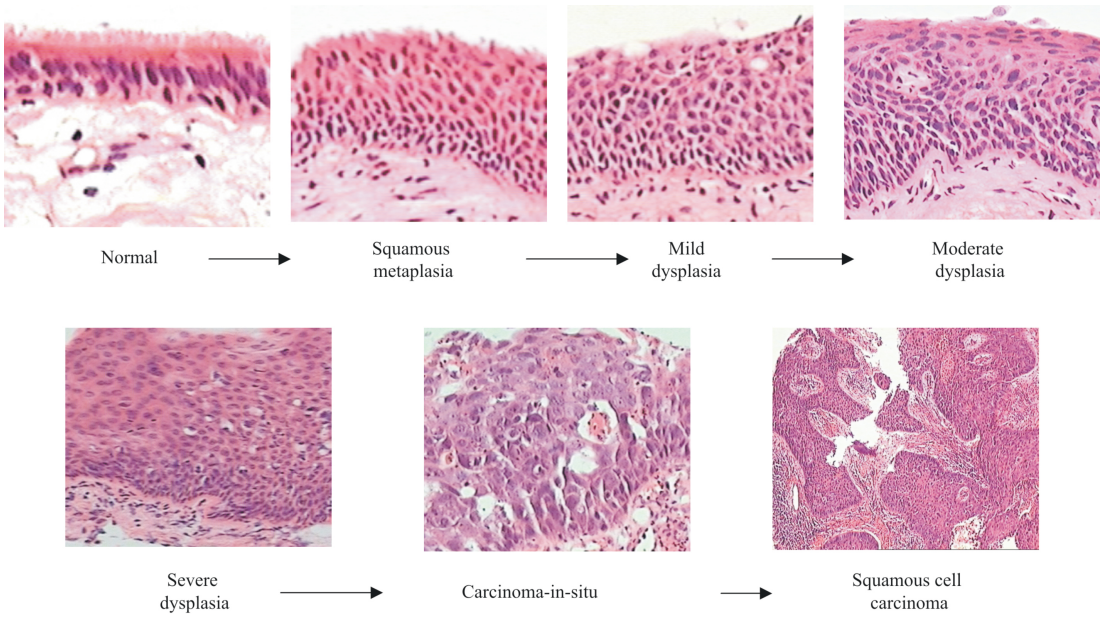
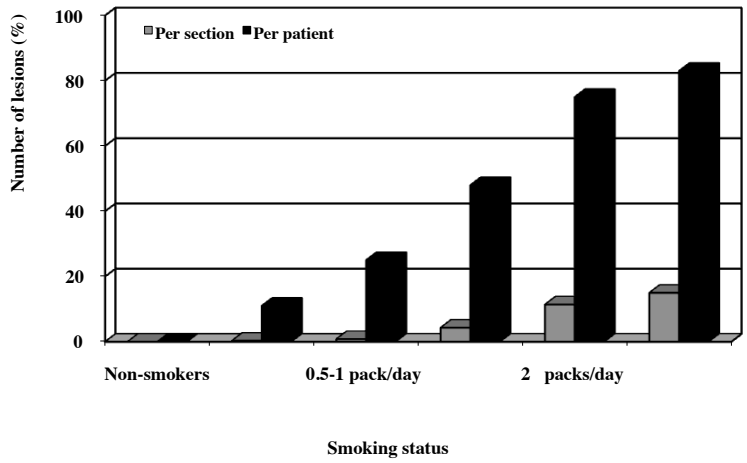


Figure 1.1 The putative pathway of development of squamous cell carcinoma of the bronchus

The prevalence of pre-invasive lesions

The seminal study by Auerbach and colleagues[12] in the 1950s examined 208 serial sections from post-mortem specimens of the bronchial tree of a cohort of smokers, non-smokers and lung cancer sufferers. Pre-invasive lesions were a frequent finding in the bronchial epithelium of smokers and patients with lung cancer and their prevalence and histological grade were related to the intensity and duration of smoking (figure 1.2)[12]. Carcinoma-in-situ was detected in 75% of the heavy (>40 cigarettes per day) smokers and 11% of the sections from these subjects. There was no “dysplasia” category in their classification of lesions, and carcinoma-in-situ was defined as “all bronchial epithelial lesions composed entirely of atypical cells and lacking cilia”. The authors accepted that there were differences between the lesions categorised as “carcinoma-in-situ” but could not differentiate the lesions further due to a lack of sensitivity in their techniques. Nevertheless, the findings suggest that pre-invasive lesion development may be a consequence of cigarette smoke exposure and could be related to the risk of lung cancer, in particular squamous cell carcinoma.

Figure 1.2 The prevalence of pre-invasive lesions in the study by Auerbach and colleagues.



The prevalence of pre-invasive lesions was addressed in a more recent study by Paris et al[13]. In 241 patients at high risk for lung cancer (as defined by the IASLC criteria) the prevalence of high-grade pre-invasive lesions was 9%. The prevalence was higher in current (12%) than former smokers (4%). The histological grade of dysplasia observed within the bronchial tree was related to the extent of tobacco exposure. There was a clear association between the presence of high-grade pre-invasive lesions and previous carcinoma of the bronchus, a history of head and neck carcinoma (odds ratio 4.0 based on 15 patients) and occupational exposure to asbestos or other carcinogens. The risk of having a high-grade pre-invasive lesion ranged from 0.2% to 90.4% and was related to the number of risk factors for carcinoma within the individual patient. A multi-centre randomised study by Häußinger[14] and colleagues using a different autofluorescence system stratified 1173 patients by their risk factors. The highest prevalence of pre-invasive lesions was in patients with abnormal sputum cytology but normal chest radiology (11.1%). Patients with previously resected carcinomas had a prevalence of 6.7% and those with radiological or clinical suspicion of lung cancer 4.6%. There were no pre-invasive lesions found in those with Chronic Obstructive Pulmonary Disease or occupational exposure to carcinogen alone.

Further data are available from the chemoprevention studies of Lam and colleagues where volunteer smokers underwent autofluorescence bronchoscopy[15]. The prevalence of carcinoma-in-situ was 1.8%, severe dysplasia 6.5%, moderate dysplasia 14% and mild

dysplasia 40%. Males appeared to have a higher prevalence of pre-invasive lesions, high-grade lesions occurring in 31% of males but only 14% of females. The overall prevalence and number of lesions per patient were lower in females even after adjustment for smoking. The reasons for this are not clear but may be related to sex-related differences in the susceptibility of the epithelium to the toxic effects of cigarette smoke.

The natural history of pre-invasive lesions

Sputum studies

The hypothesis that squamous cell carcinoma of the bronchus develops through a series pre-invasive lesions was originally inferred from the sputum studies of Saccomanno and colleagues[16]. Uranium miners, at high risk of developing lung cancers were followed using cytological analysis of serial sputum specimens for up to 10 years. The morphology of abnormal cells in sputum was considered indicative of the histology of the underlying lesion. In some patients, sputum cytology became increasingly abnormal until squamous carcinoma cells were seen. In others, the abnormalities returned to normal and carcinoma was not detected during long-term follow-up. This study has been presented as proof that squamous carcinoma of the bronchus develops from pre-invasive lesions. Although sputum is representative of the entire respiratory tract and easy to collect, the studies of Auerbach et al.[12] suggest that smokers usually carry multiple pre-invasive lesions, and therefore the fate of any individual lesion cannot be inferred from the sputum findings alone. Unfortunately, correlation with the pathology of individual lesions was not performed and so the study cannot be regarded as conclusive evidence of the pathway by which squamous cell carcinoma of the bronchus develops from pre-invasive lesions.

Animal studies

Animal studies using dogs, hamsters and rats have investigated pre-invasive lesions[17-19]. Concentrated carcinogen was applied to the tissue and the development of abnormal lesions determined either by serial biopsy or sacrifice of animals. Either the animals own bronchial tree, or transplanted human bronchial epithelium was used. On exposure to carcinogen, usually derivatives from cigarette smoke such as benzo-α-pyrene, the sequence of lesion

progression from normal epithelium through metaplasia, dysplasia and carcinoma-in-situ to invasive carcinoma was observed[19]. When the carcinogen was withdrawn, or after only a single short exposure to carcinogen, regression to normal epithelium was observed in some studies[18]. Animal studies, although useful as a model, are of limited applicability to human pathophysiology. The carcinogen levels used were higher, more concentrated and applied for a shorter time than the more chronic cigarette smoke exposure in humans. Nevertheless the results are of value as they provide experimental evidence of the pathway by which squamous cell carcinoma of the bronchus develops.

Human studies

Studies involving the follow-up of patients

The presence of pre-invasive lesions of the bronchus signifies an increased likelihood of development of carcinoma of the bronchus. Loewen et al[20] recently described a group of 169 patients from the USA undergoing surveillance using CT scanning and autofluorescence bronchoscopy. Of the patient group, 66% had pre-invasive lesions of squamous metaplasia or worse and 7% developed a lung cancer in the 3-16 month follow-up period, over half of which were adenocarcinomas. A similar group of 46 patients, reported by Pasic et al[21], were followed up using autofluorescence bronchoscopy 4-6 monthly for a median of 50 months. The number of pre-invasive lesions found within the airways was related to the patient's probability of carcinoma development. In contrast to the USA study above, 24% of this Dutch group of patients developed squamous cell carcinoma; no adenocarcinomas were found. The relationship between the individual lesions followed and the development of carcinoma was not discussed. A Japanese study followed 124 patients with serial bronchoscopies every 4-6 months. New endobronchial lesions developed in 7 patients (1 CIS, 5 squamous cell carcinomas and 1 small cell carcinoma). In 10 patients, new peripheral lesions were found (6 squamous cell carcinomas, 2 adenocarcinomas, 1 small cell and 1 large cell carcinoma). Only 3 severe dysplasia lesions developed into carcinoma-in-situ. The median time to progression was 24 months, and 80% of the lesions were stage 0 or 1. Risk factors for progression were Chronic Obstructive Pulmonary Disease, number of pack years smoked and lesion grade (i.e. high-grade lesion)[22].

A 4 year study in Italy[23] showed that 10 of 22 patients with dysplasia developed a carcinoma, 7 of which were squamous cell, during follow-up. The grade of dysplasia at baseline was related to the probability that a carcinoma would occur. There was no bronchoscopic follow-up, and so once again the relationship between individual lesions and the development of carcinoma could not be determined. Additional information is provided by the study by Sin et al.[24] from Canada, where a raised serum C-reactive protein was associated with the histologic progression of dysplasia or the development of new lesions in 50% of the study patients. These data suggest that in patients at high-risk for lung cancer, pre-invasive lesions of high grade are prevalent. The risk of development of carcinoma of the bronchus, particularly squamous cell, is related to their number and severity.

Studies involving the bronchoscopic follow-up of lesions

The literature on the natural history of pre-invasive lesions has accumulated over 50 years and is difficult to interpret. The criteria for the diagnosis and classification of pre-invasive lesions has changed twice[7,8,12,25] which makes the analysis of older studies problematic. The possibility that an individual lesion may progress to an invasive carcinoma has prompted some groups to take the view that all carcinoma-in-situ should be treated[26]. Thus in many studies carcinoma-in-situ and invasive carcinoma were joint end-points, which compromises evaluation of the natural history of high-grade lesions[27,28]. In most studies, higher grade lesions have been followed up for only very short periods of time, typically less than 6 months, and usually not as far as the development of invasive malignancy[26]. The lesions were then treated, which invalidates the follow-up data.

Despite the 2004 WHO guidelines[7], the differentiation between severe dysplasia and carcinoma-in-situ, particularly in biopsy specimens, can be difficult. It is interesting to note that the agreement between the two observers for the reporting of the histopathology has not been stated in any of the studies, nor was the quality of the biopsies assessed. This is despite known difficulties with the subdivision between mild and moderate dysplasia, between severe dysplasia and carcinoma-in-situ, and also the effect of the quality and

structural integrity of the biopsy on the interpretability of the histological features[29]. Bronchoscopic biopsy and specimen processing is known to compromise the histological appearance of some specimens. The interpretation of studies of the natural history of pre-invasive lesions is critically dependent on accurate histology, particularly if differentiation is to be made between carcinoma-in-situ and severe dysplasia.

The studies in which follow-up data on pre-invasive lesions can be found are listed in table 1.1. Given the difficulty in detecting pre-invasive lesions[30], and their relatively low frequency in the at-risk population[14], it is not surprising that the majority of studies have included patients in whom pre-invasive lesions have already been identified. Most have followed individual lesions, but some have followed the whole patient, reporting outcome in terms of the observed carcinoma, regardless of it's location or cell type. There is heterogeneity between the groups of patients included, the specific types of lesion followed, the end-points of the studies and the criteria for intervention for pre-invasive lesions.

The largest study, that of Bota et al[26], followed carcinoma-in-situ for only 3 months prior to endobronchial therapy. No further post-treatment data are given, and the efficacy of the treatments applied are not evaluated. Severe dysplasia was followed up, and found to regress in a significant proportion of patients. Only the ultimate outcome of an individual lesion was reported, limited by the length of the study, and not the specific histological or bronchoscopic features as they evolved over time. Jeanmart et al.[28] reported the outcome of individual lesions over time. As both carcinoma-in-situ and invasive carcinoma were end-points the outcome of severe dysplasia and carcinoma-in-situ are difficult to evaluate with certainty. The study by Breuer et al.[27] had similar issues, with the definition of "progression" of a high-grade lesion including lesions that had remained stable for 3 months, or progression from severe dysplasia to carcinoma-in-situ. Only 11 lesions of high-grade were identified in the study by Hoshino et al.[31] and no lesions of carcinoma-in-situ, while studies by Moro-Sibilot et al.[32], Sozzi et al.[33] and Lamy et al.[34] contribute very few high-grade lesions to the literature. The placebo arms of the chemoprevention studies of Lam et al. provide

valuable information, although follow-up was for only 6 months and there were very few high-grade lesions[35,36].

It is difficult to interpret the studies of Satoh et al.[37], Thiberville et al.[38] and Venmans et al.[39] alongside the more recent studies as the lesions were classified using the 1981 WHO criteria[25]. In the study by Venmans et al.[39] in particular, all the carcinoma-in-situ was treated, which compromised the results. Satoh et al.[37] followed 4 lesions in 3 patients, all of which developed into invasive carcinoma in up to 6 years. It is conspicuous that the high grade lesions progressed more rapidly than the low-grade lesion. The study by Thiberville and colleagues[38] followed the histological and molecular progression of a series of pre-invasive lesions, some of which were treated and some of which were not. Four lesions are shown with their detailed histological follow-up data in the study by Breuer et al.[27], but without timescales.

Tables 1.2 and 1.3 show the outcome of pre-invasive lesions from the literature. When the data from all the studies are combined it is noted that pre-invasive lesions may progress to invasive squamous cell carcinoma, and the risk is much higher for high-grade lesions than low-grade lesions. The probability of progression appears higher for carcinoma-in-situ than severe dysplasia, but this is based on the analysis of 49 carcinoma-in-situ lesions. It is not clear whether carcinoma-in-situ had indeed progressed to invasive carcinoma or persisted as carcinoma-in-situ in many of these lesions which makes interpretation of the data difficult. A significant proportion of carcinoma-in-situ and severe dysplasia lesions regress towards normal epithelium. It has been suggested that severe dysplasia is more likely to regress than carcinoma-in-situ, but the protocol of the study in question treated carcinoma-in-situ if it persisted at 3 months which invalidates the conclusions drawn[26]. It is observed that as many carcinoma-in-situ lesions remain stable as they progress to invasive carcinoma or regress towards normal, suggesting that the length of follow-up has been inadequate for many of these lesions. Combining carcinoma-in-situ and severe dysplasia together into a “high-grade” lesion category shows an equal rate of progression and regression. Low-grade

lesions have a comparatively low risk of progression and are most likely to regress to normal or remain stable.

These data are based on 165 high-grade lesions in the literature. It is questionable, given the low numbers of lesions followed-up and the heterogeneity of inclusion criteria and reporting of outcomes, whether any firm conclusions regarding the clinical management of high-grade pre-invasive lesions can be drawn. There are no clinical features that predict the outcome of a manifest lesion.

From these studies several important questions remain unanswered:

1. Does squamous cell carcinoma of the bronchus develop through a series of pre-invasive lesions of increasing histological and cytological abnormality? The length of follow-up for virtually all of the lesions in the literature is insufficient to adequately answer this question.
2. Are all pre-invasive lesions committed to the development of malignancy? There is no convincing evidence that the entire sequence of histopathological changes from normal epithelium to squamous cell carcinoma occurs in every lesion.
3. Does pre-invasive lesion development follow a predictable time-course and pattern?

Table 1.1: Studies of follow-up of pre-invasive lesions. Abbreviations: MiD Mild dysplasia, MoD Moderate dysplasia, SD Severe dysplasia, CIS Carcinoma-in-situ, ASD Angiogenic squamous dysplasia, LGL Low-grade lesion, PDT Photodynamic therapy, CRP C-reactive protein, High-risk is as defined by the IASLC criteria.

Study	Selection criteria	Patients (no.)	Male (%)	Lesions (no.)	Follow-up (months)	Lesion types included	Lesions types excluded	Comments
Lam 2001[40]	High-risk	101	61	248	6 months	Up to severe dysplasia	CIS & carcinoma	Chemoprevention study
Bota 2001[26]	High-risk	104	96	416	3-24	Up to SD	CIS treated	
Jeanmart 2003[28]	Ex-smokers	48	94	80	18-36	Up to SD	CIS & carcinoma end-points	
Breuer 2005[27]	Smokers	52	85	134	11-21	Up to SD	CIS & carcinoma end-points	
Hoshino 2004[31]	Detected lesions	50	98	99	6-17	Up to SD	None	
Moro-Sibilot 2004[32]	Detected lesions	27	89	31	25	CIS & SD	LGL and carcinoma	
Lamy 2002[34]	Detected lesions	37	NA	29	19-28	All	No SD / CIS reported	Methylation status & outcome
Sozzi 2002[33]	Detected lesions	2	100	20	48	All	CIS treated	Case study 2 patients
Keith 2000[41]	ASD only	11	NA	20	12	ASD	All others	Part of larger histology study
George 2007[42]	High risk	22	86	36	12-85	All	None	9 patients previous cancers
Lam 2004[35]	High risk	112	73	403	6	All	None	Chemoprevention study

Studies performed using pre-1999 WHO criteria for pre-invasive lesions

Study	Selection criteria	Patients (no.)	Male (%)	Lesions (no.)	Follow-up (months)	Lesion types included	Lesions types excluded	Comments
Satoh 1997[37]	SD & MiD only	3	100	4	7-72	3 SD and 1 MiD	None	Lesion follow-up case study
Venmans 2000[39]	CIS only	9	78	13	6 months	CIS + detected in follow-up	All others	PDT treatment study
Thiberville 1995[38]	High-risk	6	92	11	48	All	Some CIS treated	Molecular changes study

Studies in which lesions were treated

Study	Selection criteria	Patients (no.)	Male (%)	Lesions (no.)	Follow-up (months)	Lesion types included	Lesions types excluded	Comments
Deygas 2001[43]	CIS only	35	97	41	1-12	CIS	All others	Study of cryotherapy

Studies of resection margin CIS

Study	Selection criteria	Patients (no.)	Male (%)	Lesions (no.)	Follow-up (months)	Lesion types included	Lesions types excluded	Comments
Pasic 2005[44]	CIS only	11	82	11	11-89	CIS resection margin	All others	Resection margin lesions only

Studies in which patient outcome determined (no individual lesion data)

Study	Selection criteria	Patients (no.)	Male (%)	Follow-up (months)	Outcome	Purpose of study
Sin 2006[24]	"Dysplasia"	65	75	6	50% progression at 6 months	Relationship outcome to CRP
Ponticiello 2000[23]	High-risk with dysplasia	22	69	48	10/22 carcinoma	Relationship outcome to grade of dysplasia & p53
Pasic 2003[21]	High-risk	46	85	12-80	11/46 carcinoma	Retrospective. Relationship number of lesions to outcome

Table 1.2: Studies of follow-up of untreated pre-invasive lesions. The numbers of lesions with the given outcome are shown for each study. A range is given for studies where differentiation between outcomes cannot be determined from the data, e.g. carcinoma-in-situ stable at 3 months or progression to invasive carcinoma. The outcomes of the lesions are therefore calculated as a range. Abbreviations: MPA metaplasia, LGL low-grade lesion, HGL high-grade lesion, SD severe dysplasia, CIS carcinoma-in-situ.

	Bota 2001[26]	George 2007[42]	Jeanmart 2003[28]	Breuer 2005[27]	Hoshino 2004[31]	Moro-S 2004[32]	Lamy 2002[34]	Satoh 1997[37]	Thiberville 1995[38]	Lam 2004[35]	Lam 2003[36]	Total (numbers)	Total (%)
CIS progression	0-25/32	X	5/8	X	X	2/7	X	X	1/2	X	X	8-33/49	16-67
CIS regression	7/32	X	0/8	X	X	3/7	X	X	0/2	X	X	10/49	20
CIS stable	0-25/32	X	3/8	X	X	2/7	X	X	1/2	X	X	6-31/49	12-63
SD progression	0-8/27	X	2/9 SQC 4/9 CIS	8/25 incl CIS	2/11 SQC	1/1	X	3/3	X	0/2	0/2	20-28/80	25-35
SD regression	19/27	X	3/9	13/25	5/11	0/1	X	0/3	X	2/2	2/2	44/80	55
SD stable	0-8/27	X	0/9	4/25	4/11	0/1	X	0/3	X	0/2	0/2	8-16/80	10-20
HGL progression	0-33/59	6/36	7/17	8/25 incl some stable	2/11	3/8	X	3/3	1/2	0/2	0/2	30-63/165	19-38
HGL regression	26/59	7/36	3/17	13/25 excl CIS	5/11	3/8	X	0/3	0/2	2/2	2/2	61/165	37
HGL stable	0-33/59	23/36	7/17	4/25 excl CIS	4/11	2/8	X	0/3	1/2	0/2	0/2	41-74/165	25-45
LGL progression	6/169	0/17	11/18	9/64	1/88	X	6/29 HGL/SQC	1/1	0/9	0/145	3/105	37/645	6
LGL regression	100/169	14/17	7/18	41/64	50/88	X	0/29	0/1	3/9	102/145	61/105	378/645	59
LGL stable	63/169	3/17	0/18	14/64	37/88	X	23/29	0/1	6/9	43/145	41/105	230/645	36
MPA progression	48/152	X	5/26	13/45	X	X	X	X	X	12/68	16/29	94/320	29
MPA regression	56/152	X	0-21/26	19/45	X	X	X	X	X	0/68	8/29	83-104 /320	26-32
MPA stable	48/152	X	0-21/26	13/45	X	X	X	X	X	56/68	5/29	122-143 /320	38-45
Distant Carcinoma (number)		5	3/8				6						
Duration of follow-up (months)	3-24	12-85	18-36	11-21	6-17	25	19-28	7-72	48	6	6		

Table 1.3: Studies of follow-up of treated pre-invasive lesions. The numbers of lesions with the given outcome are shown for each study. Abbreviations: MPA metaplasia, LGL low-grade lesion, HGL high-grade lesion, SD severe dysplasia, CIS carcinoma-in-situ, PDT photodynamic therapy, CP Chemoprevention.

	Venmans 2000[39]	Lam 2002[40]	Deygas 2001[43]	Moro-Sibilot 2004[32]	Sozzi 2002[33]	Lam 2003[36]	Lam 2004[35]	Total (no. patients)	Total (%)
Distant SQC			8/35						
CIS progression	2/6	X	7/35	11/21	2/2	X		22/64	34
CIS regression	3/6	X	25/35	9/21	0/2	X		37/64	58
CIS stable	1/6	X	3/35	1/21	0/2	X		5/64	8
SD progression	3/3	X	X	1/2	0/1	0/2	0/2	4/10	40
SD regression	0/3	X	X	1/2	1/1	2/2	2/2	6/10	60
SD stable	0/3	X	X	0/2	0/1	0/2	0/2	0/10	0
HGL progression	5/9	X	7/35	3/8	2/3	0/2	0/2	17/59	29
HGL regression	3/9	X	25/35	3/8	1/3	2/2	2/2	36/59	61
HGL stable	1/9	X	3/35	2/8	0/3	0/2	0/2	6/59	10
LGL progression	1/4	1/94	X	X	1/2	3/108	3/114	9/322	3
LGL regression	3/4	55/94	X	X	1/2	74/108	73/114	206/322	64
LGL stable	0/4	38/94	X	X	0/2	31/108	38/114	107/322	33
MPA progression	X	8/20	X	X	X	5/23	15/56	28/99	28
MPA regression	X	10/20	X	X	X	10/23	34/56	54/99	54
MPA stable	X	2/20	X	X	X	8/23	7/56	17/99	17
Treatments	PDT	CP	Cryotherapy	Various endobronchial	Specific chosen	CP	CP		

Autofluorescence bronchoscopy

Autofluorescence bronchoscopy has been developed specifically to detect pre-invasive lesions[45-47]. The ability to detect lesions, particularly those of higher-grade, has enabled the study of pre-invasive lesions as a means of early identification and treatment of squamous cell carcinoma of the bronchus.

Pre-invasive lesions are small, and difficult to detect using conventional bronchoscopy[30]. In an attempt to address this problem, bronchoscopic devices have been developed which exploit differences in the fluorescence properties of normal and abnormal bronchial mucosa. They can be used to inspect the central airways and identify subtle endobronchial lesions previously not visible using conventional white light bronchoscopy. This technique cannot identify lesions beyond the range of the bronchoscope, in locations such as the distal airways and pulmonary parenchyma.

Fluorescence and tumour detection

It has been known since the early part of the last century that tissues fluoresce when exposed to light of a suitable wavelength. Tumours, both benign and malignant, alter the fluorescence properties of the tissue, facilitating their detection using this technique[48,49]. The difficulty was that within the bronchus, the intensity of fluorescence was often too low to be detected with the naked eye or was swamped by reflected light from the excitation beam. Exogenous photosensitizing fluorescent compounds, such as hematoporphyrin derivatives were used to enhance the intensity of the fluorescence pattern[50,51]. Unfortunately the photosensitizer caused transient but severe photosensitivity of the skin and consequently there was limited uptake of the technology in clinical and research practice.

Interest in fluorescence-based detection returned because subsequent technological advances in image acquisition and processing facilitated the detection of subtle differences in fluorescence characteristics without the requirement for a fluoropher. Observations in the lung have shown that dysplasia, carcinoma in situ, and microinvasive carcinoma exhibit slightly weaker red fluorescence but much weaker green fluorescence than normal tissues

when illuminated by 380-440nm wavelength (blue spectrum) light (figure 1.2)[52]. The reasons for this difference are not fully understood. In tissues, the endogenous fluorophores include collagen, nicotinamide, adenine dinucleotide, flavins and porphyrins. Cell nuclei generate relatively little fluorescence signal compared to the cell cytoplasm. In both pre-invasive lesions and carcinoma, there is an increased cell nuclear:cytoplasmic ratio, which reduces the fluorescence signal from the cytoplasm. Lesions cause thickening of the epithelium, which reduces the fluorescence from the underlying stromal collagen. Neovascularisation due to neoangiogenesis induced by the lesion cells increases the local haemoglobin concentration and thereby alters the fluorescence signal[53,54]. It is thought that the differences in autofluorescence observed in pre-invasive and malignant lesions compared to normal tissue are due to a combination of these factors, although our understanding is incomplete[52,53].

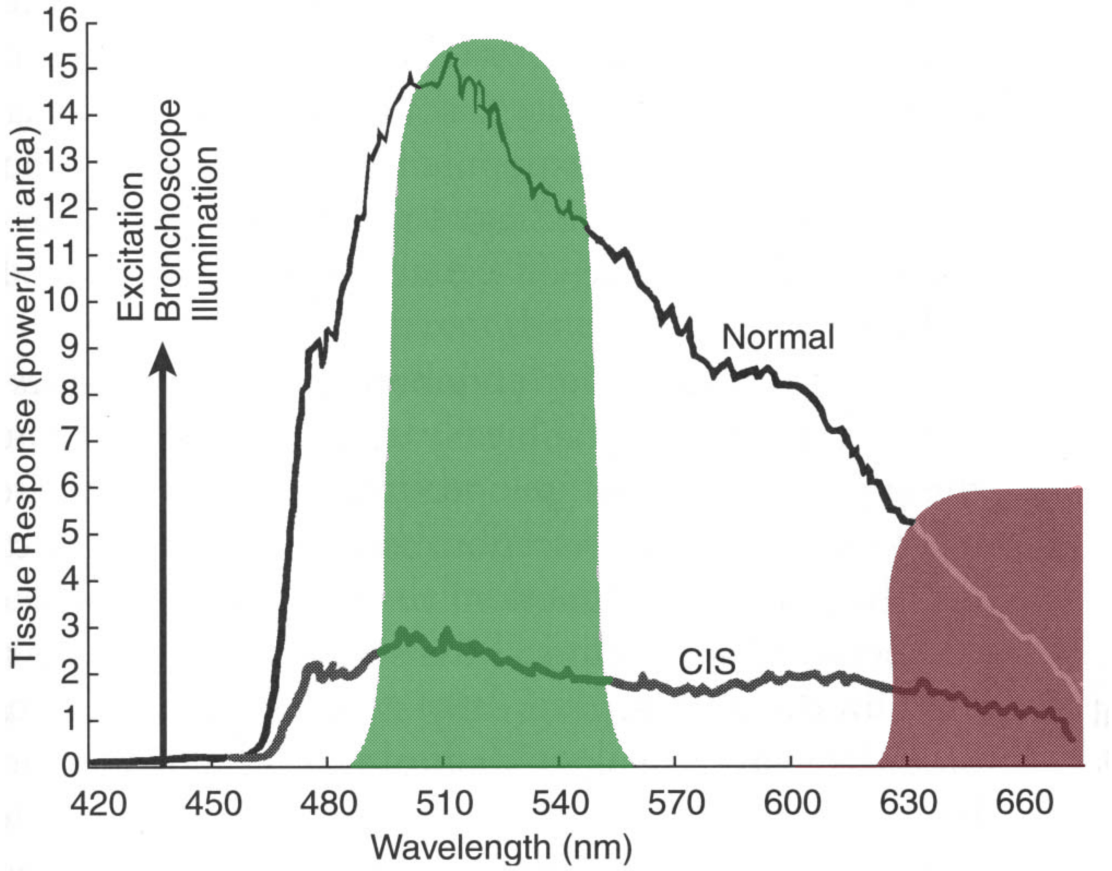
Autofluorescence bronchoscopy systems

The first bronchoscopy system to become commercially available was the light-induced fluorescence endoscopy (LIFE) device (Xillix Technologies Corporation, Vancouver, Canada)[55,56]. The bronchial tree was illuminated by blue (442 nm) light from a helium-cadmium laser, and the fluorescence images were collected by the imaging bundles of the bronchoscope. The red and green wavelengths were filtered and amplified with separate image-intensifying cameras. Their relative intensities were then measured and used to create a computer-enhanced image, which delineated the abnormal areas of fluorescence when displayed on a monitor. Fluorescence bronchoscopy was performed during the same session as conventional white light bronchoscopy, but separate light sources were required. Most of the work in this field was performed using this device, including validation of autofluorescence for the detection of pre-invasive lesions and microinvasive carcinoma.

Karl Storz Gmbh (Tuttlingen, Germany), subsequently developed a system using a conventional xenon light source, together with an optical filter fitted to the eyepiece of the bronchoscope[57]. The light source can illuminate the bronchial tree with either white light (conventional white light mode) or two bands of blue light (380 to 460 nm and 380 to 440nm,

autofluorescence mode), the mode of illumination being controlled by a switch incorporated into the bronchoscope. An optical filter transmits the red and green wavelengths together with a small part of the excitation wavelength, allowing visualization in areas of low fluorescence. The images can be viewed directly through the eyepiece of the bronchoscope or displayed on a monitor using a CCD-camera. Although the Storz system was designed to detect both autofluorescence and induced-fluorescence (using the pro-drug 5 aminolaevulinic acid) [30,31], the most commonly employed technique is autofluorescence, as it is easy to perform and avoids light sensitization.

Figure 1.3: Autofluorescence of normal epithelium and carcinoma-in-situ in the red and green wavelengths of light



Adapted from Hung J et al.[52]

Clinical studies using autofluorescence bronchoscopy

Most of the published clinical studies on autofluorescence bronchoscopy have been conducted with the LIFE device. In the majority, white light bronchoscopy was performed before autofluorescence bronchoscopy in the same session, and the abnormal areas observed with each modality were carefully documented. The ability to detect preinvasive lesions and invasive carcinoma was assessed by histological examination of biopsies taken from the bronchoscopically abnormal areas and comparison with control biopsies from bronchoscopically normal areas.

The five largest studies involving the LIFE device have reported a 1.5 to 6.3-fold increase in the detection of dysplasia and carcinoma in situ[46,55,56,58-60] (see table 1.4). Although invasive carcinomas are considered relatively easy to detect with white light bronchoscopy, some of these studies have also reported improved detection of these lesions with fluorescence bronchoscopy[46,59]. In a recent multi-centre study of patients investigated for possible carcinoma, the sensitivity of detection of high-grade pre-invasive lesions was improved by 4.25, although the false positive rate was increased by 3.56 in the same group, when compared to white light detection using the same device[59]. This suggests that despite extensive training and experience, and with the newest imaging technology, autofluorescence detection still provides benefits over white light detection for high-grade pre-invasive lesions. This benefit decreased to 1.50 when the detection of lesions from moderate dysplasia to invasive carcinoma were considered, reflecting the ability of white light bronchoscopy to detect lesions at the fully invasive carcinoma stage. In a meta-analysis of 14 studies with 15 datasets, 3612 samples from 1358 patients were analysed for lesions of moderate dysplasia or higher grade. The sensitivity of autofluorescence was 90% (range 84-93%) with a specificity of 56% (45-66%), while the sensitivity of white light bronchoscopy was 66% (58%-73%) with a specificity of 69% (57%-79%)[61].

The design of these studies has been criticized because performing a preliminary white light bronchoscopy may lead to bias in sensitivity of the subsequent fluorescence bronchoscopy[62,63]. In one large randomized study, the order in which the procedures were

performed (white light first or autofluorescence first) or the bronchoscopist performing the procedures did not influence the relative sensitivities of either modality for lesion detection[64]. In none of the studies have the abnormalities been autofluorescence negative but white light positive. The specific appearances on autofluorescence bronchoscopy do not correlate with the histopathological appearances. Although several groups have attempted to classify the abnormalities seen with the autofluorescence bronchoscope, their utility in terms of the diagnosis is not well established[46,59].

The study that showed the largest advantage for fluorescence bronchoscopy reported results that were based upon the number of biopsies that revealed abnormal histology, rather than upon the number of discrete lesions detected[46,62]. The ability to ascertain an abnormality may therefore have been exaggerated if more than one biopsy was taken from some lesions. Although these criticisms should be taken into account when interpreting the data, the broad agreement that exists among these large studies suggests that fluorescence bronchoscopy with the LIFE device genuinely facilitates the detection of moderate to severe dysplasia and carcinoma in situ.

Data in favor of fluorescence bronchoscopy have not been universal. As an example, a 1998 study from the MD Anderson Cancer Center found a lower sensitivity associated with the LIFE device[65]. The authors of this study had hoped that the LIFE device would facilitate the detection of metaplasia and dysplasia, which were endpoints in their lung cancer chemoprevention trials. However, the detection of these abnormalities was not significantly increased in 39 individuals undergoing combined white light and fluorescence bronchoscopy when compared with the results obtained in a matched group of 53 controls undergoing white light bronchoscopy alone. Biopsies from areas judged to be normal by the LIFE device yielded a similar number of metaplastic and dysplastic lesions as did those from areas judged to be abnormal. The data obtained with the LIFE device were stratified according to smoking status and history of previous smoking-related cancer, in order to establish whether abnormalities of fluorescence might reflect lung cancer risk factors other than histological abnormalities, but no correlations were found.

The apparent discrepancy between the findings of the MD Anderson study and other studies involving the LIFE device has been attributed to the lower prevalence of severe dysplasia and absence of carcinoma in situ in participants recruited into the MD Anderson study[66]. It has therefore been suggested that the LIFE device has sufficient sensitivity to detect severe dysplasia and carcinoma in situ but is neither sensitive nor specific for detecting milder degrees of dysplasia and metaplasia[66].

Clinical experience with the Storz fluorescence bronchoscope is not as extensive as with the LIFE device, but evidence suggests that both systems improve the detection of airway abnormalities to a similar degree[14,62,67,68]. One potential advantage of the Storz fluorescence bronchoscope is the shorter procedure time required for use. This was best demonstrated in a multicenter study comparing autofluorescence using both LIFE and Storz systems, in conjunction with white light bronchoscopy, in over 300 patients[67]. The diagnostic performance of each system was similar, but the time required for examination was significantly shorter with the Storz system (7.4 versus 11.7 minutes for the LIFE system). Similar reductions in examination time have been seen with the SAFE-1000 device[69].

Limitations of autofluorescence bronchoscopy

In a study of 11 healthy volunteers average age 59 years, there was a 30% inter-patient variation in the red and green intensities and a 26% variation in the red and green ratios during autofluorescence[70]. Within individuals there was also variability in the red and green autofluorescence signals due to the nature of tissues within the bronchial wall. Some variability in autofluorescence signals could be attributed to the instrument used. The variability is usually compensated by automatic colour balance within the autofluorescence system but the clinical effect of this variability is not known. The authors suggest that these factor should be taken into account when designing autofluorescence systems, but the clinical impact has never been assessed.

An important limitation of autofluorescence bronchoscopy is the high rate of false positive findings. In a large multicenter study, only 95 of 285 biopsies obtained from areas with abnormal autofluorescence detected by the LIFE device actually contained abnormal histology, yielding a positive predictive value of only 33 percent[46]. However, one study has shown that 50% of lesions with abnormal autofluorescence but normal histology carried molecular changes similar to pre-invasive lesions and invasive carcinoma[71].

In contrast, in a study from Japan autofluorescence bronchoscopy was performed immediately prior to lung resection for carcinoma[72]. The resected specimen was then subjected to detailed analysis looking for pre-invasive lesions. Autofluorescence failed to identify 8/16 (50%) of the prevalent lesions found within the resected specimen. The relationship between the autofluorescence properties of an individual lesion, the histopathology and subsequent histological outcome is not known. It is possible that autofluorescence changes in an area of bronchial mucosa reflect subtle ultrastructural changes not present in autofluorescence negative lesions. Autofluorescence changes may indicate an increased risk of developing lesions or malignancy but may just as easily signify that a lesion is indolent and unlikely to progress to malignancy. No studies have addressed this question, which will be difficult to answer until technology advances sufficiently to map lesions within the entire bronchial epithelium without the need for biopsy, as this disrupts the mucosa and may influence the long-term outcome of the lesion. Narrow band imaging has shown some promise, with a relative sensitivity for the detection of pre-invasive lesions from moderate dysplasia to carcinoma-in-situ of 3.0 compared to 3.7 for autofluorescence (no significant difference)[73]. It is not known how the vessel abnormalities seen with narrow band imaging correlate with the histologies of high-grade lesions, and therefore much work remains before the technique can be widely applied in the research context.

Clinical indications for autofluorescence bronchoscopy

The clinical applications of autofluorescence are still being evaluated but include the investigation of patients with high-grade sputum atypia with normal radiological imaging (grade of recommendation 1b), where its diagnostic yield for lung cancer may be up to

6.3%[74]. In a large multicentre study of patients using the Storz device, the sensitivity of autofluorescence for the detection of high-grade pre-invasive lesions was greatest in patients with a radiological suspicion of lung cancer, or abnormal sputum cytology but normal chest radiology[14]. Improvement in the sensitivity of lesion detection over white light of 2.5 and 2.8 respectively was obtained. Other clinical applications include longitudinal surveillance of patients with dysplasia and CIS (grade 2c), and the assessment of early central lung cancer being considered for curative endobronchial treatment (grade 2c).

It has been suggested that fluorescence bronchoscopy may be of value in identifying patients at risk of developing lung cancer. In a study of 46 patients with sputum atypia or a previous history of an aerodigestive cancer, the number of areas of abnormal autofluorescence was related to the risk of squamous cell carcinoma development over the next four years[21]. Similarly, in a separate study of 22 patients with different grades of pre-invasive lesion, combined surveillance with fluorescence bronchoscopy and annual chest CT disclosed an exceptionally high lung cancer risk in patients with severe dysplasia and CIS (54% at 2 years)[42].

In summary

Autofluorescence bronchoscopy is an effective technique for the detection of pre-invasive lesions of the bronchus. It is limited by a high false positive rate and by the inability to view the entire tracheobronchial tree. Of concern is the suggestion that not all pre-invasive lesions are visible using autofluorescence.

Table 1.4: Studies of autofluorescence.

				HGL	HGL	HGL	MoD+	MoD+	MoD+
Author	Year	Patients #	Lesions #	Sens WLB (%)	Sens AFL + WLB (%)	Relative sens	Sens WLB (%)	Sens AFL + WLB (%)	Relative sens
LIFE									
Lee[69]	2007	48	28	-	86	-	-	-	-
Moro-Sibilot[75]	2002	244	81	35	86	2.4	-	-	-
Sato[76]	2001	50	67	85	94	-	-	-	-
Shibuya[77]	2001	64	45	69	91	1.3	-	-	-
Van Rens[78]	2001	69	15	20	100	5	-	-	-
Venmans[79]	2000	114	101	57	84	1.5	-	-	-
Venmans[47]	1999	95	40	70	85	1.2	-	-	-
Vermylen[80]	1999	34	16	25	93	3.8	-	-	-
Kurie[65]	1998	39	60	43	-	-	-	-	-
Venmans[63]	1998	33	13	78	100	1.4	-	-	-
Lam[56]	1994	100	113	16	94	5.3	-	-	-
Lam[55]	1993	94	77	48	73	1.5	-	-	-
Edell[59]	2009	170	76	10	44	4.3	47	71	1.5
Chajjed[58]	2005	151	63	61	92	1.5	71	94	1.2
Chiyo[81]	2005	32	30	58	100	1.7	67	100	1.6
Ikeda[60]	1999	158	72	51	90	1.6	64	73	-
Kakihana[82]	1999	72	89	51	88	1.7	66	92	1.4
Lam[46]	1998	173	102	9	56	6.3	25	67	2.7
Ueno[83]	2007	31	29	-	-	-	54	77	-
Hirsch[64]	2001	5	75	-	-	-	22	81	3.7
Kusunoki[84]	2000	65	137	-	-	-	61	90	-
Yokomise[85]	1997	30	14	-	-	-	65	90	1.4
Ikeda[86]	1997	30	41	-	-	-	93	100	-
Total		**1901**	**1384**	**46**	**86**	**2.7**	**58**	**85**	**1.9**
SAFE1000									
Horvath[87]	1999	10	15	21	79	3.8	-	-	-
SAFE3000									
Ikeda[60]	2006	154	48	65	90	1.4	78	94	1.21
Storz									
Haussinger[62]	1999	60	7	33	83	2.8	-	-	-
Haussinger[14]	2005	53	53	58	82	1.4	-	-	-

HGL: High grade lesions, Moderate dysplasia, severe dysplasia and carcinoma-in-situ. MoD+: lesions of moderate dysplasia or worse including invasive squamous cell carcinoma. WLB: White light bronchoscopy. AFL: autofluorescence bronchoscopy. Sens: sensitivity.

The molecular biology of pre-invasive lesions of the bronchus

Introduction

The bronchial epithelium of smokers is exposed to the carcinogens in cigarette smoke. The entire tracheobronchial epithelium is exposed to carcinogen and at risk of DNA damage, a phenomenon known as "field cancerisation"[88,89]. This renders the entire tracheobronchial tree susceptible to malignant transformation and the development of carcinoma. Some carcinogens, including the benzo-α-pyrene derivatives, are known to bind to DNA and induce damage[90]. When the damaged DNA replicates and separates during mitosis gain or loss of chromosomal DNA within the daughter cells may occur. Genetic loss may inactivate tumour suppressor genes[91]. Tumour suppressor genes regulate cell growth, replication and differentiation and so prevent the uncontrolled growth of clones of cells and inhibit the development of features of malignancy such as invasion and angiogenesis. Inactivation of a tumour suppressor gene is believed to occur through a "two-hit" process, in which one allele is deleted (detectable by loss of heterozygosity) and the other is deactivated (for instance, by mutation or methylation)[92]. This frees the affected cell and subsequent clone of cells from growth and invasion control and renders it susceptible to malignant transformation.

Genetic loss has been identified at multiple chromosomal loci in lung cancers using loss of heterozygosity analysis[93-96]. Specific patterns of genetic loss are associated with each of the major histological types of lung cancer although there is considerable overlap; for instance chromosome 3p genetic loss is common in both small cell and squamous cell carcinomas[97]. It is the overall pattern of the loci of genetic loss that is more typical of specific histologies[98-100]. In squamous cell carcinoma of the bronchus genetic loss at multiple different chromosomal loci has been found, although consistent patterns have emerged[93-101].

Genetic loss in pre-invasive lesions

Genetic loss using LOH analysis has also been discovered in histologically normal epithelium from both current and former smokers but not in those who have never smoked[102,103]. There is no difference in the pattern of genetic loss between current and former smokers. The extent of this loss may vary from 1-2 loci to a pattern similar to that of

established squamous cell carcinoma. Pre-invasive lesions of both low-grade and high-grade show genetic loss. The extent of the genetic loss and the number of chromosomal regions affected is greater than in histologically normal epithelium but less than squamous cell carcinoma[104,105]. It has been proposed that the accumulation of genetic changes in histologically normal epithelium drives the progression through pre-invasive lesions of increasing abnormality to squamous cell carcinoma[106,107]. Between 10 and 20 mutations are thought to be required to generate a malignant phenotype, but it has been suggested that some genetic changes may be associated with cigarette smoke exposure, rather than malignant change[93]. This has prompted the study of genetic loss in pre-invasive lesions to determine which changes drive the progression to malignancy and the point in the pathogenesis of carcinoma at which they occur.

Pattern of genetic loss in pre-invasive lesions

Genetic loss in pre-invasive lesions has been studied using specimens resected for lung cancer[93-101]. Pre-invasive lesions of different grades were microdissected using various techniques and DNA extracted for analysis. Loss of heterozygosity was assessed using blood lymphocytes as a source of constitutional DNA. The LOH patterns of histologically normal epithelium, pre-invasive lesions and squamous cell carcinomas found within the resection specimens were compared.

It has been shown that in specimens resected for lung cancer, multiple areas of histologically normal bronchial epithelium remote from the carcinoma show genetic loss in a pattern suggesting a clonal relationship to the carcinoma[108]. In such circumstances, not only is the pattern of genetic loci showing LOH similar, but the same parental allele is lost at each locus, a phenomenon termed “Allele-specific loss”[109]. This is not universal among pre-invasive lesions, and in one study, 2 out of 8 patients were found to have different patterns of genetic or allele loss, suggesting that they are not clonally related to the carcinoma[110].

Genetic loss at 3p has been found in normal epithelium, 50-90% of dysplasia and 100% of carcinoma-in-situ and squamous cell carcinomas[109]. The loci with genetic loss were

"discontinuous", with areas of genetic loss interspersed with areas where heterozygosity was retained[105,109-111]. The extent of 3p genetic loss appears to increase in parallel with the histological grade of the lesion such that a larger number of loci show genetic loss in higher-grade lesions and carcinomas than lower-grade lesions. This suggests that LOH at 3p may progress as pre-invasive lesions progresses to invasive squamous cell carcinoma. It is concluded therefore that chromosome 3p loss is an early and frequent event in the pathogenesis of squamous cell carcinoma.

LOH at 9p has also been found in histologically normal epithelium, pre-invasive lesions of all grades and squamous cell carcinoma[104,105,112]. The frequency of lesions demonstrating genetic loss and the number of markers in which loss of heterozygosity was found was greater in higher-grade than lower-grade lesions, and was greatest in squamous cell carcinoma. LOH at 9p21 was found in 63% of dysplasia lesions and 54% of early squamous cell carcinomas with a reduced expression of p73, a molecule with similar actions to p53[113]. The extent of 9p21 LOH in dysplasia and early squamous cell carcinoma was similar to later more invasive squamous cell carcinoma. This suggests that 9p21 LOH, with consequent loss of p73 and p16 expression is an early event in the pathogenesis of squamous cell carcinoma. A greater proportion of histologically normal epithelium and squamous metaplasia lesions harbour 3p LOH than 9p LOH. It has therefore been proposed that 3p LOH occurs earlier in the pathogenesis of pre-invasive lesions than 9p LOH, but both occur as early events.

A similar pattern is seen in chromosome 8p[114], where genetic loss was found in normal epithelium, low-grade and high-grade pre-invasive lesions and squamous cell carcinoma. The proportion of lesions at each histological grade showing LOH at 8p rose with increasing histological grade. The frequency of LOH at 8p in normal epithelium and low-grade dysplasia was lower than the frequency of 3p and 9p LOH which suggests that 8p LOH is a later event than 3p and 9p LOH.

Not all genetic changes are believed to occur early in the putative scheme of the development of squamous cell carcinoma. Damage to the p53 locus on chromosome 17p detected either by sequence analysis or loss of heterozygosity has been found at much greater frequency in squamous cell carcinomas than pre-invasive lesions found in the same patient at the same time[104]. Loss of heterozygosity at 17p13 and 5q21 are found in moderate and severe dysplasia but are rare findings in normal epithelium or mild dysplasia[104] which suggests that p53 damage occurs relatively late in the development of squamous cell carcinoma.

Theoretical timescale for the natural history of pre-invasive lesions

The results of these studies have been used to construct a theoretical timescale of the molecular changes associated with pre-invasive lesion development[106]. In this scheme, 3p and 9p LOH are shown to occur in carcinogen-exposed histologically normal epithelium[105]. LOH at 8p occurs in normal epithelium and low-grade lesions, but later than LOH at 3p and 9p. LOH at 5q21 and 17p13 and the accumulation of further 3p LOH are seen as later events[38,93]. The studies from which this scheme is derived are limited as the specimens were taken at a single time-point from patients with established carcinoma. There was no follow-up of individual lesions and therefore the behaviour and outcome of the lesions under study is not known. The timescale is derived by inference from the data from these studies, and consequently is dependent on two assumptions.

The first assumption is that all pre-invasive lesions are programmed to progress to squamous cell carcinoma and that the identifiable changes drive this progression, which has not yet been conclusively proved. It is assumed that lesions at different histological grades represent points along the continuum from normal epithelium through dysplasia, carcinoma-in-situ to invasive squamous cell carcinoma. LOH identified within each lesion is considered to drive progression to the next histological grade. The second assumption is that all pre-invasive lesions progress through the putative sequence of pre-invasive lesions to squamous cell carcinoma in a predictable and consistent manner. There have been no previous longitudinal studies of pre-invasive lesion histology and behaviour, and the validity

of these assumptions is not known. The studies described above, although providing valuable insights, cannot determine whether the accumulation of genetic changes drives the behaviour of an individual lesion or identify the changes or combination of changes that drives the progression to invasion of any given lesion. Longitudinal studies of pre-invasive lesions in which serial biopsies from lesions are taken are required to obtain specimens which allow detectable genetic changes to be related directly to lesion histology, behaviour and outcome.

Only one study has attempted to identify the genetic changes associated with the natural history of pre-invasive lesions sampled by serial bronchoscopy and biopsy[38]. The results were compromised by the treatment of all high-grade lesions within the study using systemic retinoids or photodynamic therapy. None of the lesions studied progressed to malignancy. A small number of patients were studied, and data was missing for many of the lesions. The regression of pre-invasive lesions to a lower histological grade was associated with an inability to detect LOH at chromosomes 3p, 5q and 9p. This suggests that there may be a relationship between the pattern of genetic loss (LOH) within a lesion and the outcome of pre-invasive lesions.

Summary

The available data suggest that pre-invasive lesions of the bronchus, the precursors of squamous cell carcinoma, manifest genetic changes similar in pattern to that of squamous cell carcinoma. The accumulation of genetic loss, as a consequence of prolonged carcinogen exposure, may drive the progression of an individual pre-invasive lesion to malignancy, and the ultimate pattern of genetic changes may determine the outcome of that lesion. Analysis of individual lesions with close correlation of LOH analysis with clinical and outcome data may identify a pattern that drives the progression to malignancy or identify a pattern associated with the indolence or regression of lesions. The results may offer insights into the processes underlying the behaviour of pre-invasive lesions of the bronchus, and identify targets that would facilitate the identification of lesions at the highest risk of progression to malignancy. Targeted intervention can be offered to such lesions.

Part 2: The detection of bronchial lesions using autofluorescence bronchoscopy

Hypothesis

Autofluorescence bronchoscopy is more sensitive than conventional white light bronchoscopy for the detection of pre-invasive lesions and invasive carcinomas of the bronchus in patients at high risk for lung cancer.

Objectives

- Determine the sensitivity and specificity of combined autofluorescence and white light bronchoscopy for the detection of pre-invasive lesions of the bronchus.
- Determine whether autofluorescence bronchoscopy in conjunction with white light bronchoscopy is more sensitive than white light bronchoscopy alone for the detection of pre-invasive lesions and carcinoma of the bronchus.

Methods

The study protocol was approved by the University College London / University College London Hospitals committees on the ethics of research on human subjects study no 01/0147. Written informed consent to enter the study was obtained from each participant prior to study entry.

Patient selection

Patients were recruited into the study from 2 main sources:

From University College London Hospitals.

Patients at high risk for lung cancer with suspicious clinical or radiological findings were considered for the study.

Risk factors for lung cancer were defined as any of:

- a smoking history of greater than 20 pack years.
- occupational exposure to carcinogen e.g. asbestos.
- chronic airflow obstruction due to smoking.
- previous lung cancer treated with curative intent.

Suspicious clinical findings were defined as clinical symptoms or signs suggestive of lung cancer. The symptoms included cough, haemoptysis, chest pain, increasing dyspnoea, weight loss, anorexia, hoarse voice, and wheeze. Clinical signs included lobar collapse, localised monophonic wheeze, pleural or pericardial effusion and paraneoplastic neurological syndromes.

Suspicious radiological findings included mass lesion, lobar or whole lung collapse, pleural effusion and hilar or mediastinal lymphadenopathy in the context of a high risk for lung cancer. Patients with lesions considered outside the range of the bronchoscope were not considered for the study.

<u>From other referring hospitals</u>

Patients were referred specifically for assessment using autofluorescence bronchoscopy and were at high risk of lung cancer with suspicious clinical or radiological findings. The patients had undergone conventional white light bronchoscopy at the referring hospital.

The patients were referred for the following indications:

- Patients with clinical or radiological features suspicious for lung cancer but no abnormality at conventional bronchoscopy.
- Patients with abnormal sputum or bronchial cytology but no abnormality at conventional bronchoscopy.
- Patients referred for further assessment and management of pre-invasive bronchial lesions detected by conventional bronchoscopy.
- Patients referred for assessment and treatment of localised endobronchial carcinoma considered not suitable for surgical resection.
- Patients with pre-invasive lesions identified at the resection margins after resection with curative intent for lung cancer.

Exclusion criteria

- Advanced carcinoma of the lung with a poor short-term prognosis.

- Medically unfit to undergo bronchoscopy and biopsy.
- Coagulation or other disorder precluding biopsy.
- Recent pneumonia or excessive secretions that would compromise autofluorescence images.
- Failure to give informed consent.

Bronchoscopic investigation

This was performed under topical anaesthesia using lignocaine 1-4% and intravenous sedation with midazolam or propofol in accordance with the British Thoracic Society Guidelines (2001)[115]. The D-light autofluorescence bronchoscope (Karl Storz GmbH & Co. KG, Tuttlingen, Germany) was used.

Order of Procedures

1. The visible bronchial tree was inspected using white light and the images interpreted by two experienced bronchoscopists.
2. The visible bronchial tree was inspected using autofluorescence and the images interpreted by two experienced bronchoscopists and the findings noted. The bronchoscopic size and location of each abnormal lesion was estimated relative to local endobronchial landmarks such as carinae and carefully recorded. The extent, colour and surface appearance of each abnormal area of autofluorescence was recorded.
3. The bronchoscopic findings were recorded on video for later review.
4. Biopsy of bronchoscopically normal and abnormal areas.
    - *Patients where no bronchoscopic abnormality was found:* random biopsies were taken from all lobes of the lung and also from the main carina.
    - *Patients where bronchoscopic abnormalities were found:* biopsies were taken from all bronchoscopically normal lobes of the lung as controls and then from all areas identified as bronchoscopically abnormal under white light or autofluorescence.

Sampling and histopathology

Disposable "cup" forceps were used (Olympus™ Endojaw 2.0mm), a fresh pair of forceps for each bronchoscopic location biopsied to prevent cross contamination of specimens. At least 3 biopsies of 1mm diameter or greater were taken from each location to minimize bias due to sampling error. The biopsies were placed into 10% neutral buffered formalin, a fresh container for each biopsy. The biopsies were processed into paraffin wax blocks using a Leica TP1050 processing machine. This uses a 3 hour cycle involving formalin fixation, dehydration in 70%, 90% and 100% ethanol, clearing in xylene and then embedding of the biopsy in a wax block and was performed by the University College London Hospitals accredited diagnostic pathology service. The blocks were cut into 5μm sections using a microtome and stained with Gill's haematoxylin and 1% eosin Y using an automated staining machine and the standard departmental protocol.

A Histopathologist with an interest in pulmonary pathology reported the diagnostic slides using the WHO criteria 1999[8] in a blinded fashion, without any knowledge of the bronchoscopic findings. Pre-invasive lesions were subdivided into high-grade (CIS and SD) and low-grade lesions (mild and moderate dysplasia) to reflect the difficulty of making definitive histological diagnoses in biopsies that were often crushed during sampling or incomplete due to sloughing of surface layers. The slides were reviewed by a second reference pathologist with an interest in pulmonary pathology using the same criteria. In the case of disagreement over the diagnosis, a third reference pathologist from another centre reviewed the slides and a final definitive diagnosis was determined.

Subsequent management of patients

Patients with invasive carcinoma were treated according to national guidelines after discussion at the local multidisciplinary meeting. Those with pre-invasive lesions but without clinical, pathological or radiological evidence of invasive carcinoma were invited to enter a longitudinal study of the natural history of pre-invasive lesions. Patients entering this study underwent further combined white light and autofluorescence bronchoscopies at intervals. The results of these bronchoscopies are included in the present study results, although

control biopsies were not always taken.

Analysis and statistics

An initial assessment of the learning curve of the autofluorescence bronchoscope was made to ensure that the results were not confounded by the period spent learning to interpret the images. Consequently the results from the first half of the study were compared with the second half of the study. Comparison of the bronchoscopic and histological findings was made to assess the efficacy of autofluorescence bronchoscopy. Histological analysis of the entire bronchial tree is the only method by which all the pre-invasive lesions and invasive carcinomas present within the airways can be identified. This cannot be performed in a living individual, and so it is not possible to measure the true sensitivity of lesion detection for either white light or autofluorescence bronchoscopy[75].

The ability of white light and autofluorescence bronchoscopy to detect abnormal lesions was assessed by comparing the histology of biopsies obtained from sites that appeared abnormal with those from sites that appeared normal using the chi-squared test. The number of lesions identified bronchoscopically was expressed as a percentage of the total number of lesions found by biopsy of both bronchoscopically normal and abnormal areas. The change in lesion detection was used to calculate the relative sensitivity of autofluorescence and white light bronchoscopy. A two-sample test of proportions was used to separately assess the difference in autofluorescence detection of invasive carcinoma, high-grade and low-grade lesions from sites identified as having abnormal autofluorescence compared to control sites. The positive predictive values for both white light and autofluorescence were calculated. These comparisons assume that all lesions occur independently of each other and so individual lesions were required to be separated by normal mucosa. The level of significance was set at 5% and the confidence intervals were calculated using Greenwood's method.

Results

84 patients entered the study with a mean age of 63.9 years (range 35-86 years) of whom 66 were male, 18 female, and their characteristics are shown in table 2.1. All patients gave significant smoking histories and 3 had additionally been exposed to asbestos. Thirty-two patients had been diagnosed with Chronic Obstructive Pulmonary Disease. Twenty patients had previously undergone curative treatment for lung cancer.

Areas biopsied with no bronchoscopic abnormality

207 locations with normal white light and autofluorescence bronchoscopic appearances were biopsied. The results are shown in table 2.2A. Histology of 87% of the locations (180 sites) sampled showed normal bronchial epithelium or squamous metaplasia. Low-grade pre-invasive lesions were found in 10.6% (22 sites), and 2.4% (5 sites) contained high-grade pre-invasive lesions. Squamous cell carcinoma was not found in any of these locations. The negative predictive value for autofluorescence was therefore 87%.

Areas biopsied for bronchoscopic abnormality

205 areas identified as bronchoscopically abnormal either under white light, autofluorescence or both bronchoscopic modes have been biopsied. The results are shown in table 2.2B and figure 2.1. Within these sites, 30 squamous cell carcinomas, 79 high-grade lesions and 18 low-grade lesions were found. Pre-invasive lesions or invasive carcinomas were detected within 78/109 sites with white light and 127/205 sites with autofluorescence, giving positive predictive values of 72% and 62% respectively. Overall autofluorescence detected 59% (95% CI: 44-73%) more lesions (pre-invasive lesions and carcinomas) than white light, giving a relative sensitivity of 1.59.

Table 2.1: The clinical characteristics of study patients

Patient characteristics	
Median age (years)	63.9
Age range (years)	35-86
Standard deviation	9.74
Males (number)	66
Females (number)	18
Co-morbidity	
Documented COPD	32
Asbestos exposure	3
Previous carcinoma	20
Head and neck carcinoma	0
Cigarette consumption	
Current smokers (number of patients)	39
Former smokers (number of patients)	45
Mean no. cigarettes smoked	24.2 per day
Standard deviation	13.5 per day
Median no of years smoked	44 years
Mean no of years since stopped	10.5 years
Referral source	**No. of patients**
Middlesex Hospital	35
Other UK Hospitals	49
Indication for bronchoscopy	**No. of patients**
Pre-invasive lesion diagnosed at previous conventional bronchoscopy	26
Abnormal cytology found at previous bronchoscopy	17
Mass identified radiologically	15
Haemoptysis without radiological abnormality	11
Known carcinoma: assessment for treatment	7
Paraneoplastic neurological syndrome	5
Other (cough, previous carcinoma, hoarse voice)	3

Table 2.2: The bronchoscopic and histological findings.

Table 2.2A: Locations sampled with no bronchoscopic abnormality

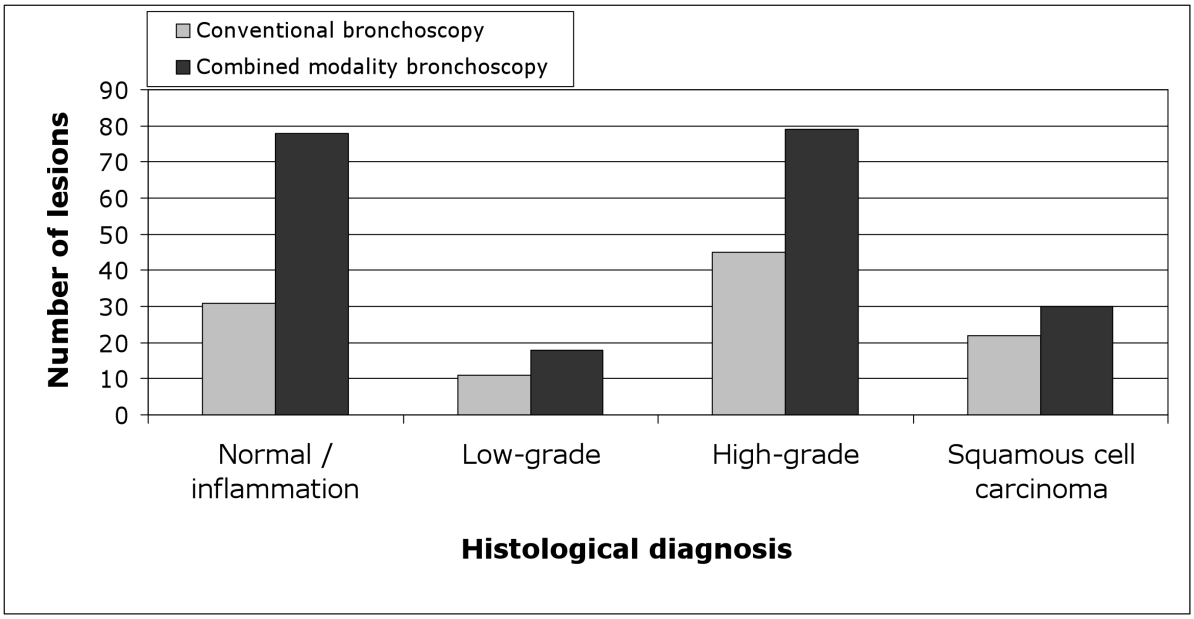
	WLB negative AFL negative	%
NAD	180	87
LGL	22	10.6
HGL	5	2.4
SQC	0	0
Total	**207**	**100**

Table 2.2B: Locations sampled for bronchoscopic abnormality

	WLB positive (All AFL positive)	WLB negative (All AFL positive)	Total AFL positive
NAD	31	47	78
LGL	11	7	18
HGL	45	34	79
SQC	22	8	30
Total	**109**	**96**	**205**

The number of patients are shown. AFL autofluorescence, WLB white light. NAD No abnormality or inflammation only. LGL Low-grade lesion, HGL High-grade lesion, SQC squamous cell carcinoma.

Figure 2.1: Histological diagnosis of areas biopsied with bronchoscopic abnormality. The number of lesions with abnormal bronchoscopic appearances and their histological diagnosis are shown.



Squamous cell carcinoma

Squamous cell carcinoma of the bronchus was diagnosed in 30 patients. Conventional white light bronchoscopy identified 22 squamous cell carcinomas, while fluorescence bronchoscopy additionally identified a further 8 carcinomas that were not apparent under white light bronchoscopy (table 2.3). The detection of invasive carcinoma by autofluorescence relative to white light bronchoscopy was enhanced by 36% (99% CI: 13% to 65%), relative sensitivity 1.36.

In 4 patients, surgery was performed with no evidence of recurrence or metastasis at 1-4 years of follow-up. Review of the autofluorescence bronchoscopy video was necessary in 2 patients immediately prior to surgery to determine the resection margin as the carcinoma could not be identified using rigid bronchoscopy. One patient was treated with radical external beam radiotherapy and remains well at 6 months. One patient has recently been treated with photodynamic therapy and remains under follow-up. In two patients who were investigated for a radiological mass, autofluorescence identified a carcinoma not visible under white light in a remote part of the bronchial tree. The patients have both undergone palliative therapy only.

Table 2.3: Patients with autofluorescence detected but white light invisible carcinoma

	Indication for bronchoscopy	Location	Histology	Stage	Treatment	Outcome
1	Pre-invasive lesion	LLL apical	SQC	1a	Surgery	Well 1 year
2	Radiological lesion	LLL	SQC	1a	Radical radiotherapy	Well 6 months
3	Abnormal cytology	RML	SQC	1a	Surgery	Well 2 years
4	Abnormal cytology	RLL	SQC	1a	Surgery	Well 4 years
5	Abnormal cytology	RLL apical	SQC	1a	Surgery	Well 4 years
6	Previous carcinoma	RUL	SQC	1a	PDT	Awaited
7	Radiological lesion	LLL	SQC	4	Palliative	Palliative
8	Radiological lesion	L main	SQC	4	Palliative	Palliative

Pre-invasive lesions

High-grade lesions

Of 205 locations biopsied, 79 have shown HGLs all of which were positive to autofluorescence, but only 45 were visible using white light. There were 5 HGLs identified in biopsies from bronchoscopically normal mucosa. Autofluorescence enhanced the detection by of high-grade pre-invasive lesions by 76% (99% CI: 56% to 90%), relative sensitivity 1.76

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Low-grade lesions

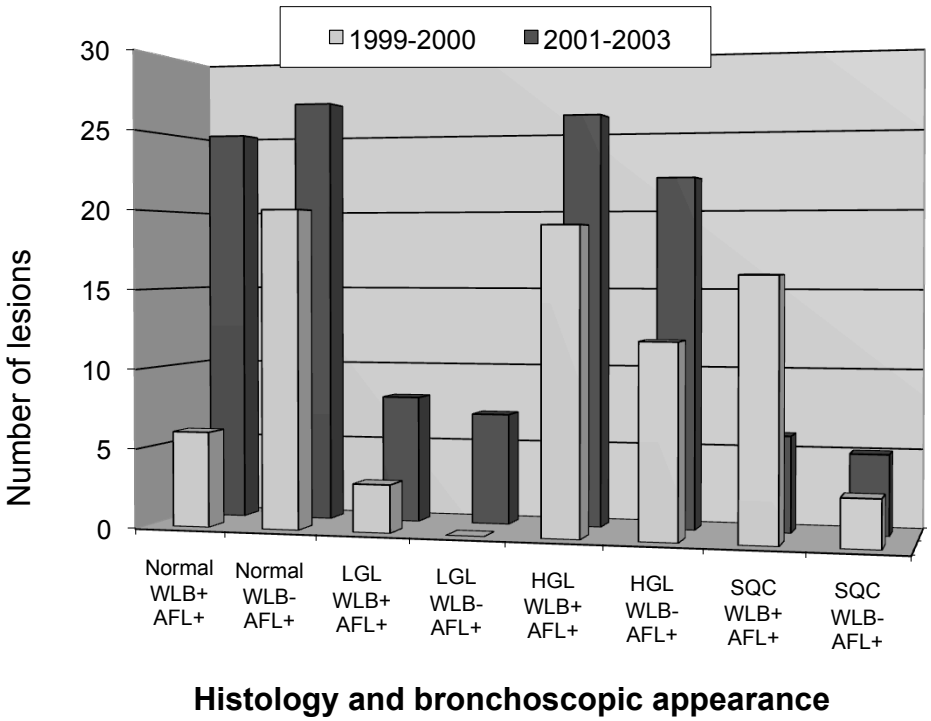
LGLs were identified in 18 sites under autofluorescence, while 11 appeared abnormal under white light. Autofluorescence enhanced the detection of low-grade lesions by 64% (99% CI: 23% to 93%), relative sensitivity 1.64, in lesions visible bronchoscopically. There were 22 LGLs identified in biopsies from bronchoscopically normal mucosa. This shows that 55% of the detected LGLs were not visible bronchoscopically to either white light or autofluorescence. When the detection of LGLs by autofluorescence or random mucosal biopsy are compared, there was a decrease in the number of lesions identified by autofluorescence by 1.9% (99%CI: -9.4% to 5.7%), relative sensitivity 0.98.

Effect of the learning curve

In order to assess whether the results could be affected by a potential learning curve of the instrument, the results of bronchoscopies performed during the period 1999-2000 were compared to the period 2001-2003 (figure 2.2). 42 patients were bronchoscoped in the period 1999-2000 and 42 patients were bronchoscoped in the period 2001-2003. There was no statistical difference in the sensitivity of detection of squamous cell carcinoma, high-grade lesions or low-grade lesions between the two time periods (p=0.38 using Chi-squared test with continuity correction).

Figure 2.2: Comparison of lesion detection rates 1999-2000 vs. 2001-2003

The detection rates of lesions using combined modality autofluorescence and conventional white light bronchoscopy are compared. WLB white light bronchoscopy, AFL autofluorescence, LGL low-grade lesion, HGL high-grade lesion, SQC squamous cell carcinoma. The time period 1999-2000 is shown in grey, and 2001-2003 in black.



Discussion

84 patients with risk factors and clinical features suspicious for lung cancer underwent bronchoscopy with conventional white light followed by autofluorescence. In the study patients, 84 HGLs and 40 LGLs were found. Autofluorescence improved the sensitivity of detection of high-grade lesions by 76% (relative sensitivity 1.76). The sensitivity of detection of bronchoscopically visible LGLs was improved by 64% (relative sensitivity 1.64) but there was little overall impact on the detection of LGLs as 55% of the LGLs identified were found in random biopsies of bronchoscopically normal mucosa. The addition of autofluorescence to white light bronchoscopy enabled the detection of 8 squamous cell carcinomas that were not identifiable under white light alone.

1. Validity of the results

a. The learning curve

The present study shows that there was no significant learning curve with the autofluorescence bronchoscope used and excludes the possibility of bias due to this effect. This is primarily due to the quality of the images produced and the clear differentiation between normal and abnormal provided by the autofluorescence equipment. The bronchoscopies were performed by a physician with a special interest in early bronchial mucosal lesions in a research setting with sufficient time for careful inspection of the bronchial mucosa. The images were interpreted by 2 experienced bronchoscopists. Although this provides the best possible analysis and interpretation of the findings, this may have introduced bias through over diagnosis of minor abnormalities, or the identification of subtle abnormalities that would not be visible in usual clinical practice with such a device. The high observed false positive rate lends support to this idea.

b. False positive results

In the present study combined white light and autofluorescence bronchoscopy produces a false positive rate of 38%. This is comparable with previous studies using autofluorescence, although most were performed using the LIFE device, which suggests that there was no significant over-diagnosis bias[46,55,64,86]. The high false positive rate observed in the present,

and in previous studies, may be due to a number of different confounding factors. Excessive mucus or secretions within the bronchial tree, or mucosal damage due to trauma from the bronchoscope may produce false positive autofluorescence appearances. The presence of blood, from trauma to the bronchus from the bronchoscope, endobronchial bleeding or epistaxis from per-nasal bronchoscopy may contribute to the false positive rate. Squamous metaplasia and mucosal inflammation, common in both smokers and patients with chronic obstructive pulmonary disease, may result in sufficient mucosal disease to generate abnormal autofluorescence. Although traumatic mucosal damage and mucus can usually be identified under white light bronchoscopy, inflammation and squamous metaplasia can be difficult to identify, closely resembling abnormal mucosa. Consequently using current technology a significant false positive rate is considered inevitable when using autofluorescence detection systems.

Molecular changes similar to HGLs have been found in 21/26 patients exhibiting abnormal autofluorescence patterns but no evidence of histopathological HGL, but none of 21 patients with normal bronchoscopic and histopathological appearances[71]. The areas of abnormal autofluorescence were histopathologically metaplasia, inflammation and LGL, and showed fewer chromosomal changes than HGLs, but more changes than normal epithelium. It is uncertain whether abnormal autofluorescence represents an area of mucosa at higher risk of lesion or malignant disease development, or is a marker of damaged or inflamed epithelium. There are no follow-up studies of autofluorescence positive, histopathologically normal epithelium. Molecular studies would compare the allelotypes and expression patterns of bronchoscopically normal and abnormal mucosa with normal histology. Bronchoscopic follow-up of these false positive areas would allow the determination of their malignant potential.

The results of the present study suggest that all bronchoscopic areas appearing abnormal under autofluorescence require histological confirmation. Some histologically normal bronchial mucosa has abnormal autofluorescence, and some histologically abnormal mucosa has normal autofluorescence. The relationship between autofluorescence

appearances, histopathology and outcome of an individual area of bronchial mucosa is not known. Studies are required to further define the meaning of an autofluorescence abnormality.

It is not clear whether histology is the best standard against which autofluorescence should be evaluated, and further studies are required. The act of biopsy disrupts the mucosa and alters the biology of the epithelium. This is reflected in the persistent of autofluorescence abnormality for up to a year in any area of traumatised or biopsied bronchial mucosa. Sampling methods that avoid disruption of the mucosa, including optical or light-based sampling techniques, would mitigate this problem, but are as yet not ready for research or clinical practice.

c. Order of the procedures

White light bronchoscopy was performed immediately before autofluorescence in all the study patients and so the bronchoscopists were aware of the white light findings during the autofluorescence bronchoscopy. There is a potential source of bias from this approach. Previous studies of autofluorescence have shown that there is no difference in the detection rates of pre-invasive lesions due to the order of the procedures, although these studies were performed using the LIFE bronchoscope[63]. The method used avoids the potential over diagnosis bias as white light bronchoscopy was expected to be less sensitive and was performed before the more sensitive autofluorescence. This prevented the autofluorescence images affecting the interpretation of the white light appearances. Ideally a separate pair of bronchoscopists would have interpreted the white light and then the autofluorescence appearances, but limitations on the personnel available within the study precluded this approach.

2. The detection of pre-invasive lesions

a. Squamous cell carcinoma

Autofluorescence facilitated the detection of 8 squamous cell carcinomas that were not identified using conventional white light bronchoscopy. This allowed a curative resection to

be performed for the micro-invasive carcinoma in 4 of the patients. One patient was successfully treated with radical radiotherapy and another with Photodynamic Therapy with curative intent. In the studies presented here, autofluorescence will be used as the major detection modality for HGLs, with the development of micro-invasive carcinoma as an important study endpoint. The results suggest that autofluorescence is effective as a diagnostic tool for the detection of micro-invasive carcinoma, significantly improving the detection rate over conventional white light bronchoscopy by 36%.

b. High-grade lesions

The detection of HGLs was improved using combined modality white light and autofluorescence bronchoscopy over conventional white light bronchoscopy by 76% (relative sensitivity 1.76). This suggests that autofluorescence is of value in the detection of HGLs. The improvement in lesion detection is lower than in previous studies that used the LIFE device, but comparable to the smaller initial studies using the Storz device (table 1.4). The improvement in sensitivity may have been compromised by the study protocol, in which careful inspection of the bronchial mucosa may have enhanced white light detection rates. This is supported by the finding that 58% of the HGLs detected were also visible using the conventional white light mode and is higher than the 13-29% detection rate in older studies[116].

The false negative rate of 2.4% using random biopsies as the control suggests that some lesions were missed during combined modality white light and autofluorescence bronchoscopy. The exact size of this error cannot be measured, as the true prevalence of both carcinoma and HGLs cannot be determined without detailed histological analysis of the entire bronchial tree. This can only be achieved by lung resection immediately after bronchoscopy or at post-mortem examination. In a study using the LIFE device in which autofluorescence was performed immediately prior to lung resection for carcinoma, autofluorescence detected only 50% of high-grade lesions and squamous cell carcinomas in the bronchial tree[72]. In the present study, the comparison with control biopsies provides an estimate of the positive and negative predictive value of autofluorescence. Together, the

findings suggest that the combination of white light and autofluorescence using the study protocol is an effective and sensitive method for the detection of high-grade pre-invasive lesions of the bronchus, and can be applied to surveillance studies of bronchial pre-invasive lesions.

The prevalence of pre-invasive lesions found in random biopsies from bronchoscopically normal areas of the bronchial tree, at 10.6% for LGL and 2.4% for HGL, shows that the overall prevalence for pre-invasive lesions is higher than was detected using autofluorescence in this selected group of patients. This finding is in keeping with the "field cancerization" hypothesis[89], and suggests that there may be two distinct populations of pre-invasive lesions, autofluorescence positive and autofluorescence negative. As the autofluorescence negative lesions are impossible to detect other than fortuitously using biopsy, they are impossible to study at this time. However, they may influence the outcome of the patients under study and so techniques to allow their detection are needed. This finding may explain the incidence of carcinoma development in locations remote from the lesions under observation in the previous studies of pre-invasive lesion outcome (see part 1: Pre-invasive lesions of the bronchus, and table 1.2).

c. Low-grade lesions

In 84 patients at high-risk for lung cancer with suspicious clinical features, from whom 205 bronchoscopically abnormal locations were sampled, only 18 LGLs were found. This represents a significantly lower prevalence than would be expected based on previous studies. The sensitivity of LGL detection appeared to be improved using combined modality white light and autofluorescence compared to conventional bronchoscopy, but 22 LGLs were found in 207 sites with no bronchoscopic abnormality under white light or autofluorescence. Combined modality white light and autofluorescence bronchoscopy identified 18/40 LGLs and random biopsies 22/40 LGLs. This suggests that the targeting of autofluorescence abnormalities does not improve the detection of low grade lesions when compared to random biopsies. The one previous study of autofluorescence in patients with LGLs only is in agreement, as lesion detection was unaffected by the addition of autofluorescence using the

LIFE device to conventional white light bronchoscopy[65]. These data suggest that autofluorescence is a successful tool for the identification of HGLs and squamous cell carcinoma of the bronchus, but less effective for the detection of LGLs.

The differentiation of HGLs, LGLs, false positive findings and invasive carcinomas is not currently possible using current autofluorescence technology. This means that all abnormal bronchoscopic appearances require histological confirmation. The significance and outcome of pre-invasive lesions, both those visible bronchoscopically and those not detected by autofluorescence is not established, and require a longitudinal study of bronchoscopic and histological changes throughout the bronchial tree. Ideally, the bronchoscopic findings would be correlated with detailed analysis of the histology of the visible bronchial mucosa, but such a study is currently not technically feasible in vivo.

In summary, combined modality white light and autofluorescence bronchoscopy significantly improves the sensitivity of HGL and invasive carcinoma detection, but has at best a limited impact on LGL detection. This technology is currently the most sensitive method of HGL and invasive carcinoma detection, and is suitable for use in longitudinal studies of pre-invasive bronchial lesions.

Part 3: The natural history of pre-invasive lesions of the bronchus

Hypothesis

Squamous cell carcinoma of the bronchus develops from carcinogen-exposed bronchial epithelium through a series of pre-invasive lesions of increasing histological and cytological abnormality.

Objective

The objective of this study was to

- Confirm that squamous cell carcinoma of the bronchus develops from carcinogen-exposed bronchial epithelium through a series of pre-invasive lesions of increasing histological and cytological abnormality.
- Determine the time-course of pre-invasive lesion development
- Determine whether pre-invasive lesions are committed to the development of malignancy

Methods

The study protocol was approved by the University College London / University College London Hospitals committees on the ethics of research on human subjects study no 01/0148. Written informed consent to enter the study was obtained from each participant prior to study entry and confirmed at each bronchoscopy.

Patient selection

Patients were identified from “The detection of bronchial lesions using autofluorescence bronchoscopy” study discussed in part 2 who had pre-invasive lesions of the bronchus but no clinical or radiological evidence of invasive carcinoma. At the follow-up out-patient appointment after autofluorescence bronchoscopy the study was offered to all eligible patients. No patient declined participation, although some patients withdrew from the study during the follow-up period (see results).

Exclusion criteria

- A diagnosis of invasive carcinoma of the lung at presentation. However, patients who have previously undergone curative treatment for invasive carcinoma of the lung and who have no clinical or radiological evidence of disease persistence or recurrence were eligible for the study.
- General medical conditions precluding bronchoscopy or affecting short-term prognosis.
- Specific respiratory disease affecting ability to tolerate bronchoscopy.
- Recent pneumonia or excessive secretions that would compromise autofluorescence images.
- Abnormalities of coagulation precluding safe bronchial biopsy.
- Refusal or inability to give informed consent.

Investigation

The overall protocol of the study is shown in figure 3.1. The significance and implications of pre-invasive lesions and the options for management were discussed with each patient in an outpatient setting.

Clinical assessment

i. History and Examination

At first presentation to the study a full clinical history was taken and a clinical examination performed. A past medical history of malignant disease (including specifically lung cancer and head and neck carcinoma), smoking related disease, chronic airflow disease including asthma and disorders that would affect the suitability for bronchoscopy were obtained. Symptoms and signs compatible with lung cancer, paraneoplastic syndromes or chronic airflow obstruction were specifically noted. A detailed smoking history was taken, including the number of cigarettes smoked per day, the age at which smoking started and the date of smoking cessation if applicable. An occupational history focusing on occupational exposure to carcinogen was obtained.

ii. Investigations

Blood tests Full blood count, urea, creatinine and electrolytes and coagulation studies were performed to ensure the patient was safe to undergo bronchoscopy

Spirometry was obtained to assess for chronic airflow obstruction as a risk factor for lung cancer and to assess suitability and safety of bronchoscopy.

CT scan of the thorax. To exclude a mass lesion or other evidence of carcinoma including metastases or lymphadenopathy.

Positron Emission Tomogram To identify pulmonary masses, hilar and mediastinal lymphadenopathy, and metastases not identified by CT scan of the thorax.

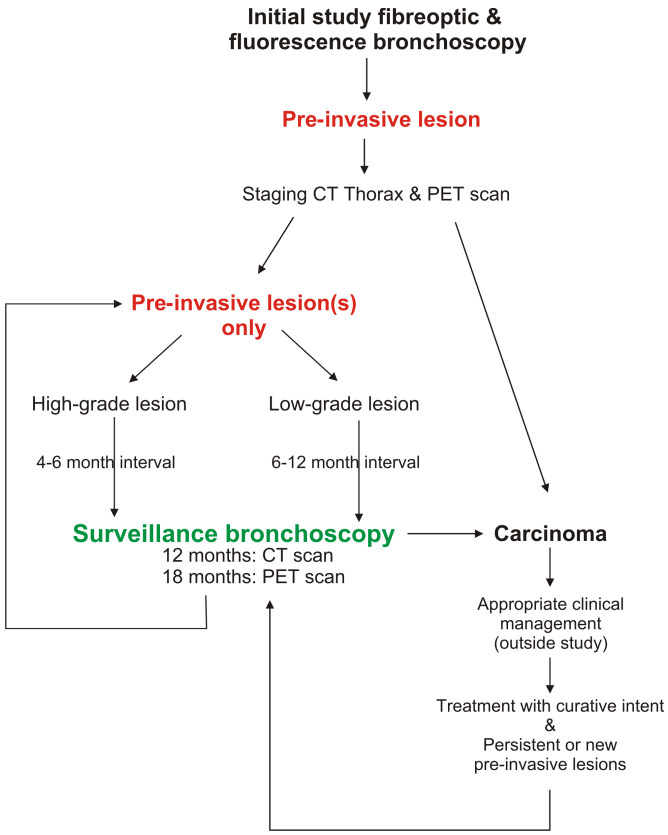
Bronchoscopic procedures

An initial study bronchoscopy was performed to confirm the presence of pre-invasive lesions of the bronchus and exclude carcinoma of the bronchus with the greatest possible confidence. This occurred within 4 months of "The detection of bronchial lesions using autofluorescence bronchoscopy" study bronchoscopy discussed in part 2 and was performed under a general anaesthetic. A rigid bronchoscope was inserted through which the fibreoptic bronchoscope was passed. This approach was adopted to facilitate thorough inspection of the entire visible tracheobronchial tree and provide conditions under which the most accurate and best quality biopsies could be obtained. Those patients with pre-invasive lesions but without evidence of carcinoma underwent continued follow-up of the lesions.

Follow-up bronchoscopies were performed under sedation with topical anaesthesia to the bronchial tree in accordance with the British Thoracic Society Guidelines (2001)[115]. In some patients, a general anaesthetic was used due to previous difficulties with sedation or due to patient preference. The frequency of follow-up bronchoscopies was determined by the histological grade of the most abnormal lesion found at the previous bronchoscopy:

Low-grade pre-invasive lesion (Mild-moderate dysplasia)	6-12 monthly
High-grade lesion (carcinoma-in-situ or severe dysplasia)	4 monthly

Figure 3.1: The programme of the study of pre-invasive lesions of the bronchus



At each follow-up bronchoscopy, the results of previous biopsies were reviewed, and the locations to be biopsied determined. All pre-determined areas of the bronchial tree were re-biopsied, as well as any areas of new white light or autofluorescence abnormality. The procedure was performed using the same protocol as the initial bronchoscopy, except that control biopsies were not taken. The histopathology results returned from the laboratory 2 weeks later. At that time the results were discussed within the research team, and the date of the next bronchoscopy booked.

During follow-up of pre-invasive lesions, to ensure that distant or co-incident carcinomas had not developed, a CT scan of the thorax was performed annually, and a PET scan was obtained every 18 months. In circumstances where the development of carcinoma was suspected, for instance a change or worsening of symptoms, worsening chest radiological appearances such as a new mass or lobar collapse, new abnormalities on study PET or CT

scan or increasing suspicion of invasion in the biopsies from high-grade lesions, the patient underwent further detailed CT scans of the thorax, a PET scan and a full clinical assessment for carcinoma and staging.

Bronchoscopy protocol

White light bronchoscopy

All visible areas of the bronchial tree were inspected. The location, size and appearance of areas abnormal bronchoscopically were noted and recorded on video for later review.

Autofluorescence bronchoscopy

Performed after white light bronchoscopy. The location, size and appearance of areas abnormal bronchoscopically under autofluorescence were noted and recorded on video for later review.

The bronchoscopic appearances were reviewed by 2 bronchoscopists experienced in both conventional and autofluorescence bronchoscopy and a consensus was reached regarding the findings. An example of the bronchoscopic appearances is shown in figure 3.2. The abnormalities were carefully mapped onto a bronchial tree diagram (figure 3.3) for later reference during surveillance bronchoscopies. Sites identified as bronchoscopically abnormal either under white light or autofluorescence were selected for sampling.

Follow-up of patients began in 1999 and the bronchoscopic appearances were recorded in clinical records by Dr Jeremy George. This author established the study protocols, the Ethical Committee approvals and Research and Development approvals in 2001. Formal systematic data collection was commenced at this time. The data collection was performed by this author from 2001-2004. After the end of this author's fellowship in 2004 data was collected by Dr Jeremy George and Sister Bernadette Carroll using protocols and recording mechanisms established by this author in 2001. Analysis of all the data was performed by this author.

Figure 3.2: Example bronchoscopic appearances from a patient with carcinoma-in-situ of the bronchus. The white light (A) and autofluorescence appearances (B) are shown.

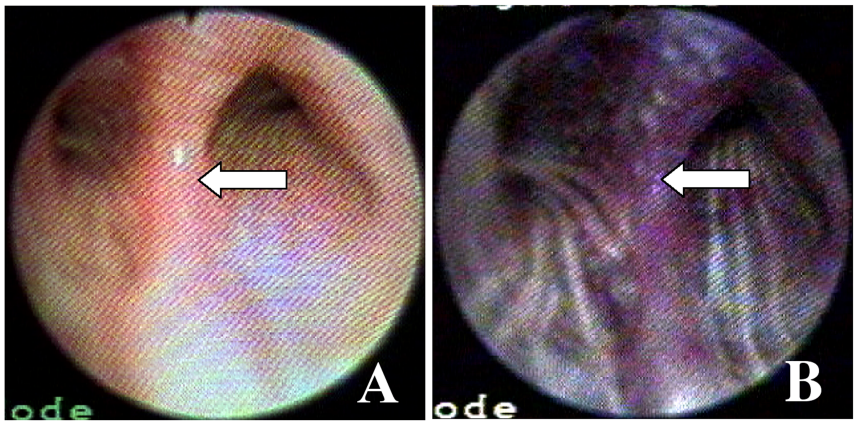


Figure 3.3: A example of a study bronchoscopy report

Fluorescence Study Report

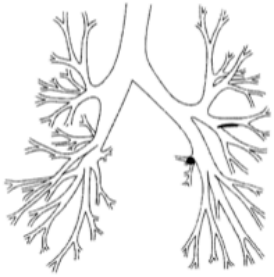
name: date of study study no:

Abnormalities

Site	White light	Fluorescence	Nature
Lingula	x NAD w/3	✓	no polyp. sup division mauve abn AFL.
LLL apical	✓	✓	dark purple shiny AFL. Occluded.
			sup division mauve AFL

Biopsies

Site	A: Abnormal C: Control	F: Formalin G: OCT gel	Destination & Comments (UC or Cam)
Lingula	A	F2 G2 f2	
LLL	A	F3 G3 f3.	
		F G f	
		F G f	
		F G f	
		F G f	
		F G f	



Brush site: Lingula & LLL apical

Wash site: Lingula & LLL apical.

Blood ☑ EDTA 3

Serum 2.

Sputum X.

Sample acquisition

i. Bronchoscopy samples

- Control biopsies were obtained systematically from sites appearing normal under both white light and autofluorescence at the initial bronchoscopy only. Each lobe of the lungs that did not show an abnormality was sampled.
- Biopsies of bronchoscopically abnormal areas. Distal locations with bronchoscopic abnormalities were biopsied prior to more proximal areas to reduce cross-contamination of biopsy material.
- Bronchial wash cytology of bronchoscopically the most abnormal area. The site was washed with 30ml of 0.9% saline solution and the aspirate divided in half. Half of the sample was stored in a plain Universal container. The other half was transferred to a bottle containing PreservCyt™ (Cytyc Corp™, Boxborough, USA).
- Bronchial brush cytology of bronchoscopically the most abnormal area. The site was brushed with a standard protected cytology brush under direct vision through the bronchoscope. Smears were prepared onto 4 slides, three of which were plain glass and the other a ChemMate™ Capillary gap immunohistochemistry slide (Dako™, Ely, UK). Two plain glass slides were sprayed with Cytofixx® cytological fixative (Cellpath PLC™, Hemel Hempstead, UK) and allowed to air dry. The other two slides (one plain glass slide and the ChemMate slide) were allowed to air dry briefly and then rapidly frozen to -80°C.
- Biopsy of the most abnormal area bronchoscopically. This was judged subjectively on a combination of the extent of the bronchoscopic abnormality and the severity of the mucosal abnormality assessed by the visual appearances including mucosal nodularity, irregularity, dark autofluorescence images, contact bleeding.

A minimum of 3 biopsies per location of at least 1mm diameter each judged visually were obtained to minimize bias from sampling error. The first 2 biopsies were placed into 10% neutral buffered formalin, a fresh container for each biopsy. The third was immediately placed into Tissue-Tek OCT gel (Raymond Lamb Ltd™, Eastbourne, UK) and frozen on dry ice to -80°C. For each bronchoscopic location biopsied, the sequence of 2 biopsies to

formalin and 1 to frozen was repeated until sufficient material for analysis (judged subjectively) had been collected or the lesion was completely obscured by blood and debris from biopsy. Disposable "cup" forceps were used (Olympus^{TM} Endojaw 2.0mm), a fresh pair of forceps for each location for biopsy to prevent cross contamination of specimens. Cup forceps minimise trauma to the biopsy, and maintain the integrity of the epithelium better than toothed forceps.

ii. Sputum

This was collected by the patient into a plain Universal container over the 24 hours prior to attendance for bronchoscopy. This was sent to the Cytology department, University College London Hospitals, processed onto slides and stained with the Papanicolau technique. The cytology was reviewed by a Consultant Cytopathologist. The remainder of the sample was stored frozen for future studies.

iii. Blood

12ml of fresh whole blood was collected into tubes coated with EDTA (Becton-Dickinson Vacutainer™, USA) as an anticoagulant and stored at -80°C to be used later as a source of reference DNA for molecular studies. A further 8ml of blood was collected into Serum Separation Tubes (Becton-Dickinson Vacutainer™, USA), spun down and the serum supernatant removed and stored at -80°C for use in later studies.

Sample Processing

Each biopsy was placed on an acetate strip for identification and immediately fixed in 10% neutral buffered formalin for 4 hours. The biopsies were then processed into paraffin wax blocks using a Leica TP1050 processing machine. This uses a 3 hour cycle involving formalin fixation, dehydration in 70%, 90% and 100% ethanol, clearing in xylene and then embedding of the biopsy in a wax block and was performed by the University College London Hospitals accredited diagnostic pathology service. The blocks were cut into 5μm sections using a microtome and stained with Gill's Haematoxylin and 1% Eosin Y using an automated staining machine and the standard departmental protocol. A Histopathologist with

an interest in pulmonary pathology reported the diagnostic slides using the WHO criteria 1999 (figure 3.4), and the slides were reviewed by a second reference pathologist with an interest in pulmonary pathology using the same criteria. In the case of disagreement over the diagnosis, a third reference pathologist from another centre reviewed the slides and a final definitive diagnosis was determined.

The biopsies frozen in OCT were stored in plastic boxes in a -80°C freezer. Care was taken to ensure that the biopsies did not thaw at any point during specimen transport from the bronchoscopy suite to the freezer. The bronchial brushing slides that had been frozen during the bronchoscopy were stored along with the biopsies in the freezer. The bronchial washings that had been collected into PreservCyt™ were processed onto plain glass slides using a Thinprep™ cytology preparation machine using the standard manufacturer's protocols.

Management of the outcomes of pre-invasive lesions

Invasive carcinoma: On detection of invasive carcinoma the patient was offered treatment with curative intent. For those patients treated with curative intent, any remaining pre-invasive lesions continued under follow-up. In those patients in whom cure was not achieved, further appropriate management was offered outside the study.

Regression to normal epithelium: was defined as the finding of normal histology from an area of a previous pre-invasive lesion during 2 successive bronchoscopies. Despite regression, lesions were maintained under surveillance in the present study.

Recording of study data

Central to this project, and to the co-ordination between the clinical findings and the results from the scientific projects is the recording of the bronchoscopic appearances, the location of lesions in the bronchial tree, the number of biopsies obtained from each lesion and the histopathological results. This was achieved in 3 ways.

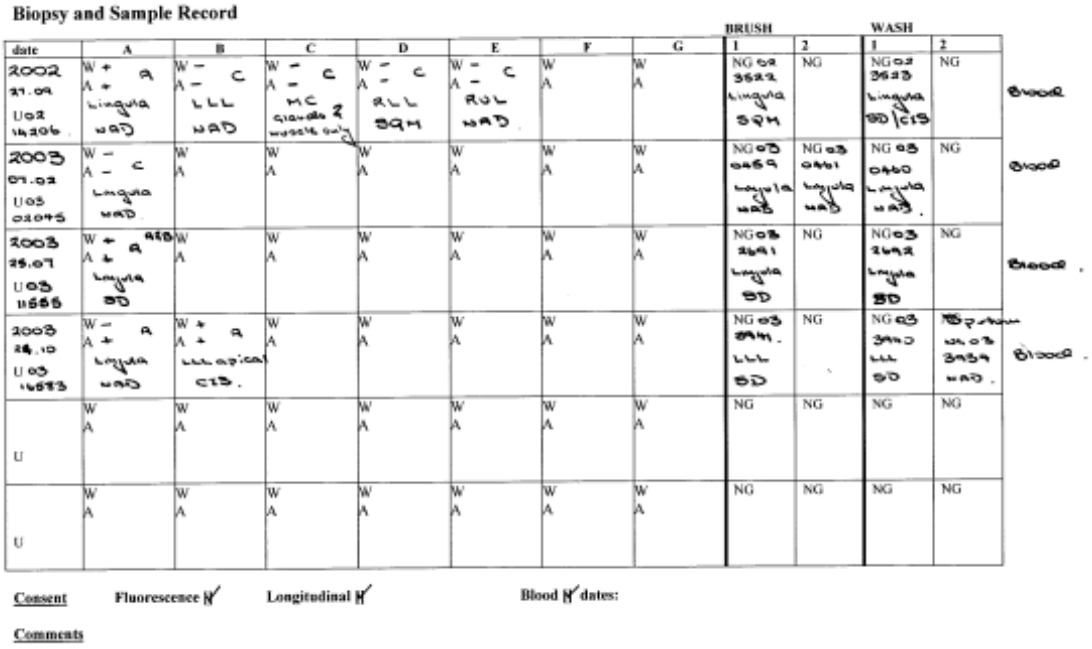
Figure 3.4: The WHO Criteria for the diagnosis of pre-invasive lesions of the bronchus 1999[8]

Abnormality	Thickness	Cell size	Maturation/orientation	Nuclei
Mild dysplasia	Mildly increased	Mildly increased Mild anisocytosis, pleomorphism	Continuous progression of maturation from base to luminal surface Basilar zone expanded with cellular crowding in lower third Distinct intermediate (prickle cell) zone present Superficial flattening of epithelial cells	Mild variation of N/C ratio Finely granular chromatin Minimal angulation Nucleoli inconspicuous or absent Nuclei vertically oriented in lower third Mitoses absent or very rare
Moderate dysplasia	Moderately increased	Mild increase in cell size; cells often small May have moderate anisocytosis, pleomorphism	Partial progression of maturation from base to luminal surface Basilar zone expanded with cellular crowding in lower two thirds of epithelium Intermediate zone confined to upper third of epithelium Superficial flattening of epithelial cells	Moderate variation of N/C ratio Finely granular chromatin Angulations, grooves and lobulations present Nucleoli inconspicuous or absent Nuclei vertically oriented in lower two thirds Mitotic figures present in lower third
Severe dysplasia	Markedly increased	Markedly increased May have marked anisocytosis, pleomorphism	Little progression of maturation from base to luminal surface Basilar zone expanded with cellular crowding well into upper third Intermediate zone greatly attenuated Superficial flattening of epithelial cells	N/C ratio often high and variable Chromatin coarse and uneven Nuclear angulations and folding prominent Nucleoli frequently present and conspicuous Nuclei vertically oriented in lower two thirds Mitotic figures present in lower two thirds
Carcinoma in situ	May or may not be increased	May be markedly increased May have marked anisocytosis, pleomorphism	No progression of maturation from base to luminal surface; epithelium could be inverted with little change in appearance Basilar zone expanded with cellular crowding throughout epithelium Intermediate zone absent Surface flattening confined to the most superficial cells	N/C ratio often high and variable Chromatin coarse and uneven Nuclear angulations and folding prominent Nucleoli may be present or inconspicuous No consistent orientation of nuclei in relation to epithelial surface Mitotic figures present through full thickness

i. A paper record was created for each patient. The case history was recorded along with the patients demographic data. Bronchoscopy and fluorescence study reports, histopathology, radiology and PET scan reports and all clinical correspondence was filed. This record was used to co-ordinate the clinical data and histological data. Figure 3.5 shows the correlation chart of the histology and bronchoscopic findings from a sample patient record which was used to determine the bronchoscopic locations under surveillance at each bronchoscopy

ii. A computer database was written using Filemaker pro 5.5™ for Macintosh™ and Windows™. This mirrored the paper record and contained each patients demographic data, and the bronchoscopic results listed by bronchoscopy. The site from which each individual biopsy was taken, the histology result and the physical location in which the biopsy was stored was recorded for use in the scientific projects. The form of the biopsy (fresh frozen or formalin fixed to block) was listed, and the history of the biopsy (e.g. defrosted from frozen to block) was recorded.

Figure 3.5: A correlation chart of biopsies and bronchoscopic findings



Biopsy and Sample Record

								BRUSH		WASH	
date	A	B	C	D	E	F	G	1	2	1	2
2002 27.09 U02 14206	W + A + Lingula WD	W − A − C LLL WD	W − A − C MC clavulin 2	W − A − C RLL SQM	W − A − C RUL WD	W A	W A	NG 02 3622 Lingula SQM	NG	NG 02 3623 Lingula BD C13	NG
2003 07.02 U03 02045	W − A − C Lingula WD	W A	W A	W A	W A	W A	W A	NG 03 0459 Lingula WD	NG 03 0461 Lingula WD	NG 03 0460 Lingula WD	NG
2003 25.07 U03 11555	W + A + Lingula BD	W A	W A	W A	W A	W A	W A	NG 03 2691 Lingula BD	NG	NG 03 2692 Lingula BD	NG
2003 28.10 U03 14883	W − A + Lingula WD	W + A + LLL apical C13.	W A	W A	W A	W A	W A	NG 03 3941 LLL BD	NG	NG 03 3943 LLL BD	NG 03 3959 WD
U	W A	W A	W A	W A	W A	W A	W A	NG	NG	NG	NG
U	W A	W A	W A	W A	W A	W A	W A	NG	NG	NG	NG

Consent Fluorescence ✓ Longitudinal ✓ Blood ✓ dates:

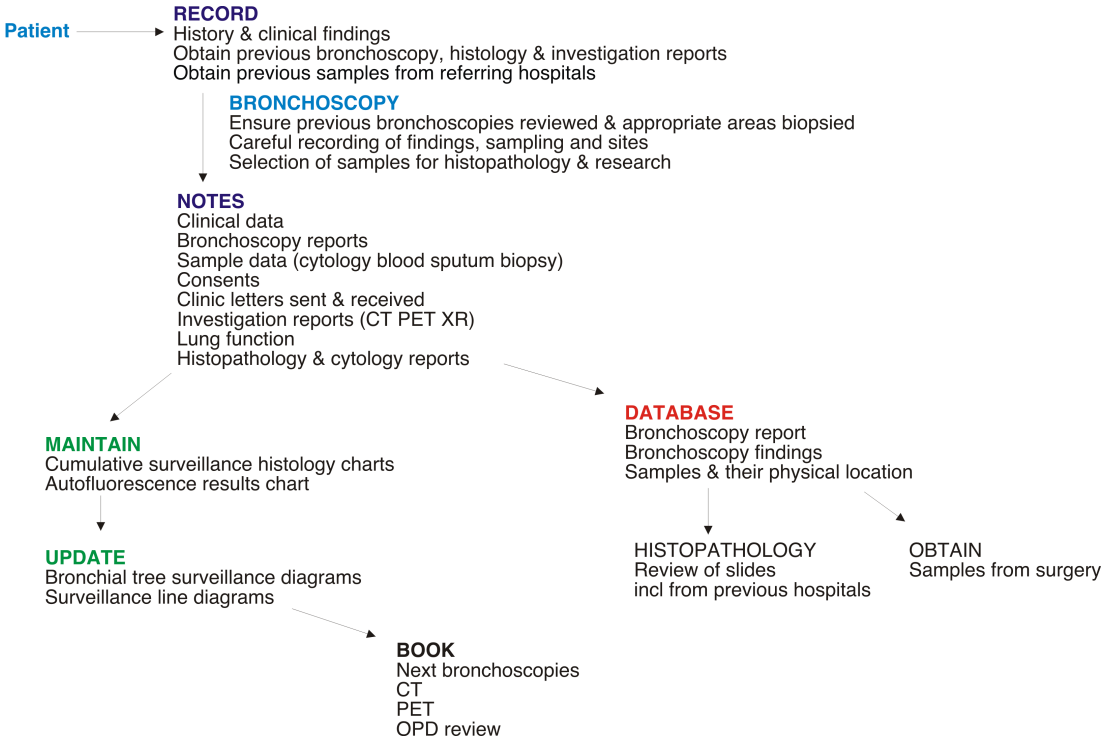
Comments

iii. From the records a timeline was generated for each patient showing each lesion, its bronchoscopic location of origin and the histology of that lesion over time. This was used to generate the cumulative results and charts.

Project Management

In addition to the above, each patient's past history was obtained form the referring hospitals where available. Samples from biopsies taken at these hospitals were obtained and processed so that they were available for the scientific studies. Figure 3.6 shows the process of the management for the surveillance study.

Figure 3.6: The management structure of the project



Analysis

The behaviour of each pre-invasive lesion under follow-up was observed over a period of time. Statistical methods of analysis could not be applied to this study design and this was discussed with the Department of Medical Statistics, University College London Hospitals. For the purposes of evaluation the WHO classification (figure 3.4) was subdivided into high-grade lesions (carcinoma-in-situ and severe dysplasia) and low-grade lesions (mild and moderate dysplasia). This was due to the difficulties in differentiating carcinoma-in-situ from severe dysplasia and moderate dysplasia from mild dysplasia in bronchial biopsies using the WHO criteria[8]. The bronchial biopsies were often traumatised during the biopsy procedure and processing, such that epithelial lesions were often difficult to orientate, and therefore sub-classify.

Results

There were 26 patients (23 male) median age 66.5 years (range 49-75 years) studied for a median of 31 months (range 14 to 110 months). The histological diagnoses of the 60 pre-invasive lesions studied are shown in figure 3.7. The lesions occurred in the same lung in 10 of 16 patients with HGLs and both lungs in the remainder. The lesions were distributed in all lobes of the lungs, with no specific predilection for any lobe or bronchoscopic location including carinae or bronchial walls.

Observations from the follow-up of pre-invasive lesions

Pre-invasive lesions evolve into squamous cell carcinoma

There was progression to invasive squamous cell carcinoma in eight lesions in seven patients (lesion L1, L2, L3, L11, L20, L24, L28, L44). The progression occurred between 4 and 34 months after the first detection of a lesion. In all except lesion L24 the first detected abnormality was HGL, and no lesions progressed directly to carcinoma from LGL. Lesion L24 was first noted at LGL, followed by normal epithelium, with subsequently HGL until the development of squamous cell carcinoma. None of the lesions progressing to invasive squamous cell carcinoma showed any evidence of histological regression during their time-course.

Pre-invasive lesions progress to high-grade histology

Progression from a LGL to a HGL was observed in one lesion (L14) over 8 months. This lesion later regressed to normal epithelium.

Pre-invasive lesions show apparent regression to normal epithelium

Histological regression to normal epithelium was observed in 17 lesions. The highest grade of abnormality attained was HGL in eight of the lesions (L6, L14, L16 possibly treatment related, L22, L23, L31, L33, L37) and LGL in nine lesions (L15, L34, L35, L39, L40, L48, L58, L59, L60). The 12 LGL with no evidence of HGL development remained at LGL for less than 18 months and 9 out of 12 regressed to normal epithelium. One lesion remained at low-grade for 38 months and then regressed to normal.

In 4 lesions, LGL was detected between 12 and 50 months after the regression of a previous pre-invasive lesion (L22, L33, L52, L56). HGL was found in 3 lesions (L54, L55, L57) 28 months after the regression of LGLs. The remaining ten lesions that regressed showed no evidence of a pre-invasive lesion. No specific features predicted regression in an individual lesion.

Pre-invasive lesions with unchanged histology

There was no change in the histological diagnosis of 21 HGLs (L4, L5, L7, L8, L9, L10, L12, L13, L17, L25, L26, L27, L30, L32, L36, L41, L42, L43, L45, L46, L47) followed for between 6 and 58 months (median 14 months). Some of the lesions were affected by treatment to a nearby carcinoma thus invalidating further follow-up data (e.g. L12 and L17). Lesion L30 was observed for 58 months without evidence of carcinoma development or of regression.

New pre-invasive lesions develop at any location

As part of the study protocol, randomly selected bronchoscopically normal areas were biopsied as "controls". In 10 of these areas that had been normal histologically (L6, L13, L25, L26, L31, L48, L49, L50 L53, L56), pre-invasive lesions were identified at later bronchoscopies. None progressed to invasive carcinoma, although 3 of the lesions (L6, L31, L48) apparently regressed to normal epithelium while the remainder did not change histology.

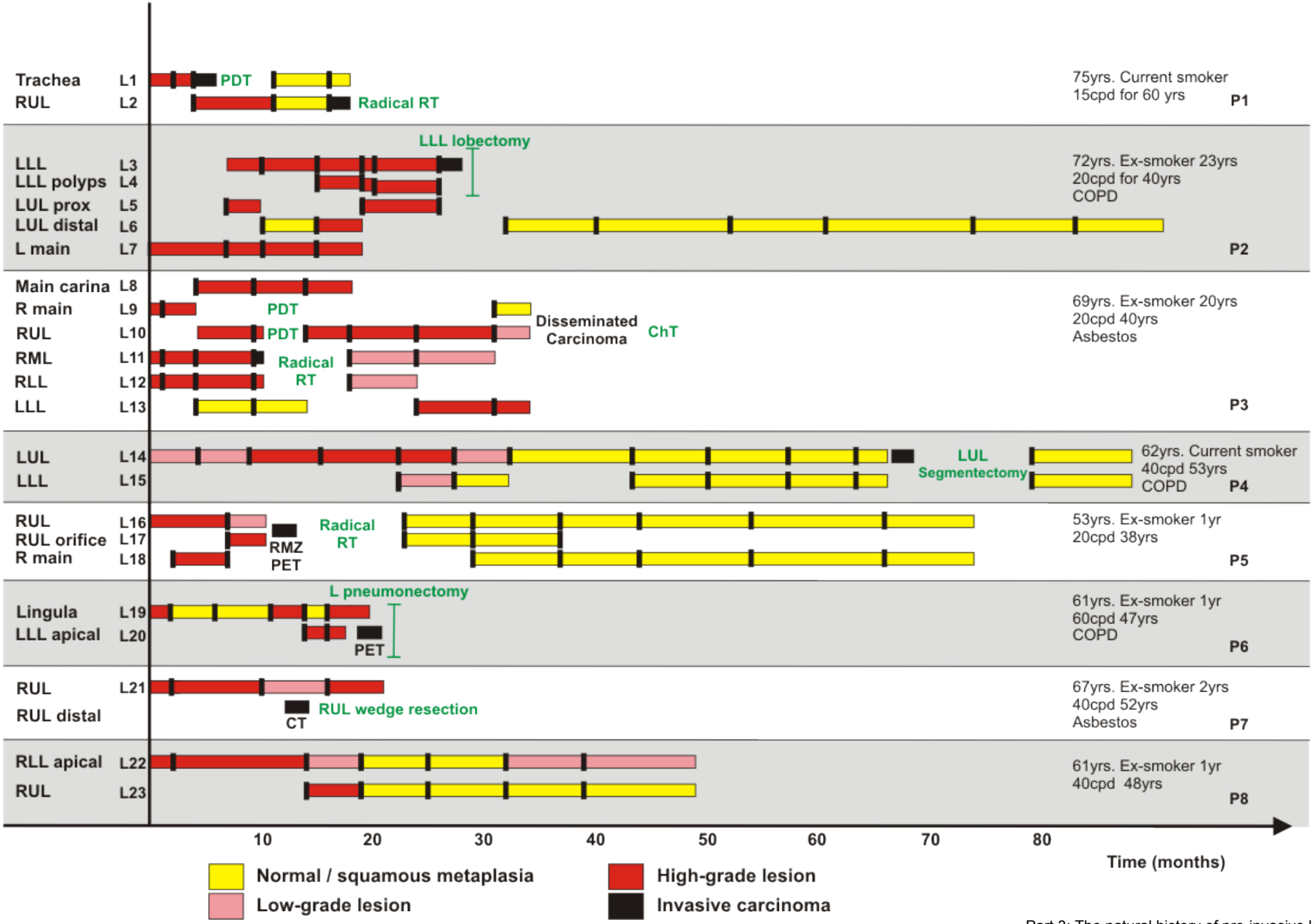
During the course of the study 24 new lesions were identified using autofluorescence bronchoscopy (19 HGL L2, L3, L4, L5, L6, L8, L10, L13, L17, L18, L20, L23, L26, L27, L29, L31, L32, L41, L47, and 5 LGL L49, L50, L53, L56, L57) in locations that had been bronchoscopically normal and not previously biopsied. Their natural history was not different to those lesions that were detected during earlier bronchoscopies.

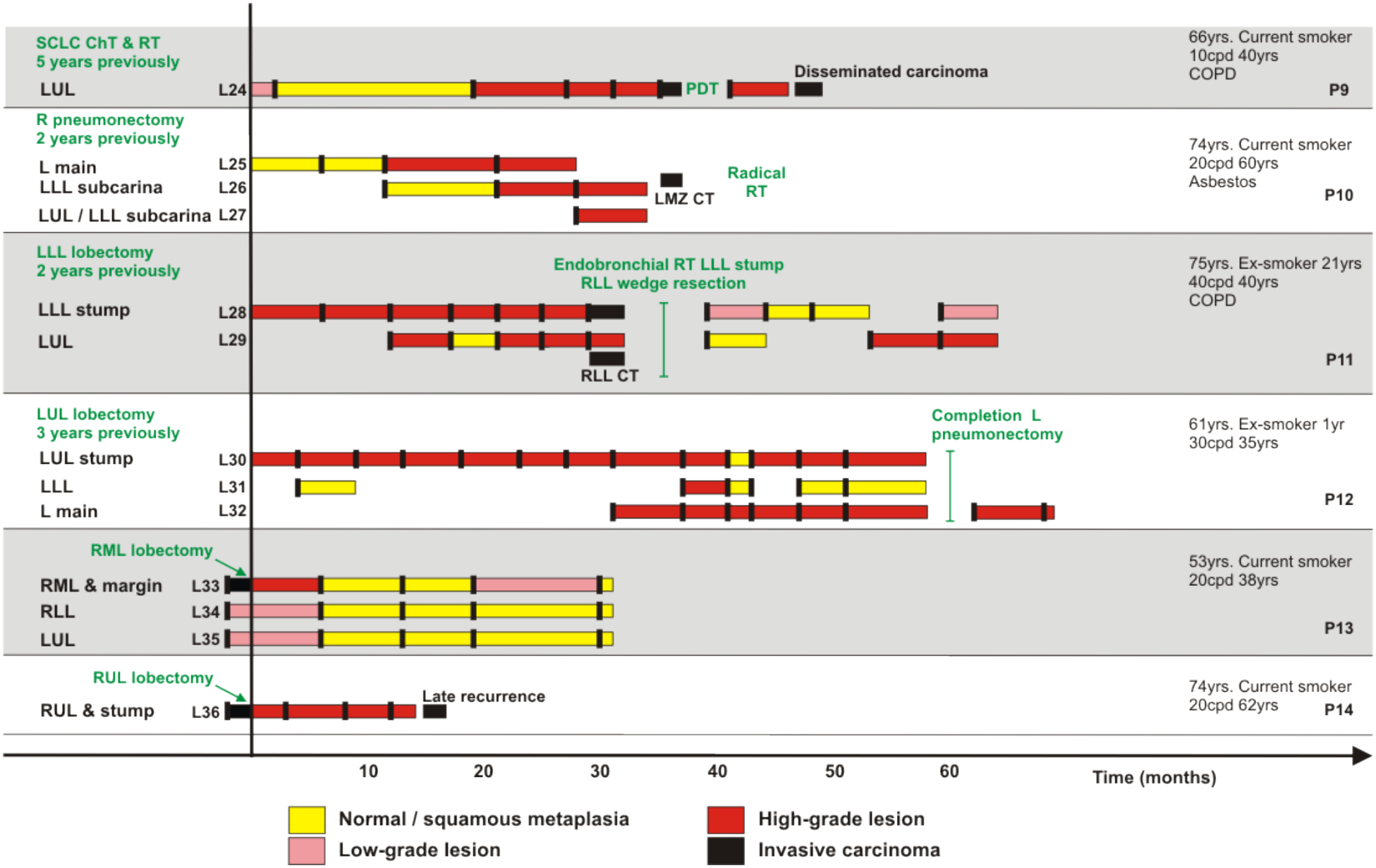
Pre-invasive lesions may not show consistent histological appearances in serial biopsies

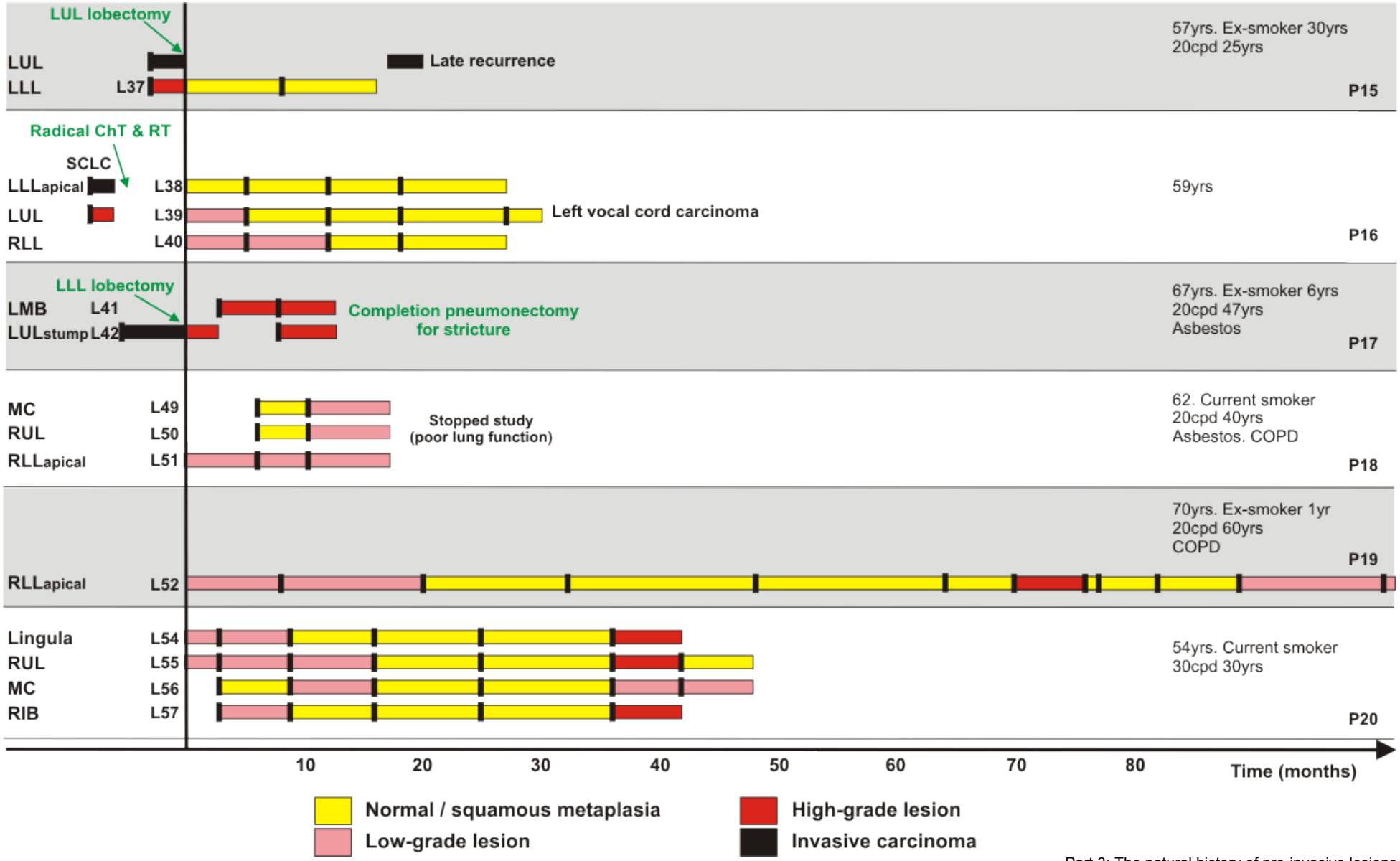
In lesions L2, L19, L22, L29 and L30 during serial biopsy, high-grade histology appeared to be interspersed with normal histology. Similarly, lesions L21 and L43 showed high-grade histology with episodic low-grade histology. Bronchoscopically (judged by their autofluorescence appearances) all the lesions were too large to be completely removed by bronchoscopic biopsy, although multiple biopsies were taken from each lesion at each bronchoscopy.

Figure 3.7: The histopathological natural history of pre-invasive lesions of the bronchus

This shows the evolution of lesions during the serial bronchoscopies of 26 patients with the UCL patient number shown on the right hand side. Biographical data regarding the patients are given and the results of all the bronchoscopies in all the patients are shown. Each bronchoscopic location with an abnormality is shown by a horizontal bar with the bronchoscopic location given on the left hand side. The colour of the bar represents the histopathological diagnosis at the bronchoscopic location at the time-point on the x-axis. The timing of each bronchoscopy in the timeline of the patient is shown by a black vertical bar. Treatments, interventions and disseminated carcinoma are shown as text. A gap within the horizontal bar shows that no biopsy was taken at that time-point. A step in the horizontal bar indicates that the following lesion whose histology is described was not in the exact bronchoscopic location as the preceding lesion, but within the same lobe. The P number refers to the UCL study number and the L number is the lesion number referred to in the text. Abbreviations: PDT photodynamic therapy, RT radiotherapy, LLL left lower lobe, ChT chemotherapy, LUL left upper lobe, RUL right upper lobe, RML right middle lobe, RLL right lower lobe, R main right main bronchus, L main left main bronchus, apical the apical segment of lobe in question, SCLC small cell lung cancer, cpd cigarettes per day, COPD chronic obstructive pulmonary disease.


Trachea L1
RUL L2
PDT
Radical RT
75yrs. Current smoker
15cpd for 60 yrs
P1
LLL lobectomy
LLL L3
LLL polyps L4
LUL prox L5
LUL distal L6
L main L7
72yrs. Ex-smoker 23yrs
20cpd for 40yrs
COPD
P2
Main carina L8
R main L9
RUL L10
RML L11
RLL L12
LLL L13
PDT
PDT
Radical RT
Disseminated Carcinoma
ChT
69yrs. Ex-smoker 20yrs
20cpd 40yrs
Asbestos
P3
LUL L14
LLL L15
LUL Segmentectomy
62yrs. Current smoker
40cpd 53yrs
COPD
P4
RUL L16
RUL orifice L17
R main L18
RMZ PET
Radical RT
53yrs. Ex-smoker 1yr
20cpd 38yrs
P5
Lingula L19
LLL apical L20
L pneumonectomy
PET
61yrs. Ex-smoker 1yr
60cpd 47yrs
COPD
P6
RUL L21
RUL distal
CT
RUL wedge resection
67yrs. Ex-smoker 2yrs
40cpd 52yrs
Asbestos
P7
RLL apical L22
RUL L23
61yrs. Ex-smoker 1yr
40cpd 48yrs
P8
10
20
30
40
50
60
70
80
Time (months)
Normal / squamous metaplasia
Low-grade lesion
High-grade lesion
Invasive carcinoma


SCLC ChT & RT
5 years previously
LUL
L24
PDT
Disseminated carcinoma
66yrs. Current smoker
10cpd 40yrs
COPD
P9
R pneumonectomy
2 years previously
L main
LLL subcarina
LUL / LLL subcarina
L25
L26
L27
LMZ CT
Radical
RT
74yrs. Current smoker
20cpd 60yrs
Asbestos
P10
LLL lobectomy
2 years previously
LLL stump
LUL
L28
L29
Endobronchial RT LLL stump
RLL wedge resection
RLL CT
75yrs. Ex-smoker 21yrs
40cpd 40yrs
COPD
P11
LUL lobectomy
3 years previously
LUL stump
LLL
L main
L30
L31
L32
Completion L
pneumonectomy
61yrs. Ex-smoker 1yr
30cpd 35yrs
P12
RML lobectomy
RML & margin
RLL
LUL
L33
L34
L35
53yrs. Current smoker
20cpd 38yrs
P13
RUL lobectomy
RUL & stump
L36
Late recurrence
74yrs. Current smoker
20cpd 62yrs
P14
10
20
30
40
50
60
Time (months)
Normal / squamous metaplasia
Low-grade lesion
High-grade lesion
Invasive carcinoma


LUL lobectomy
LUL
LLL
L37
Late recurrence
57yrs. Ex-smoker 30yrs
20cpd 25yrs
P15
Radical ChT & RT
SCLC
LLLapical
L38
LUL
L39
Left vocal cord carcinoma
RLL
L40
59yrs
P16
LLL lobectomy
LMB
L41
LULstump
L42
Completion pneumonectomy
for stricture
67yrs. Ex-smoker 6yrs
20cpd 47yrs
Asbestos
P17
MC
L49
RUL
L50
RLLapical
L51
Stopped study
(poor lung function)
62. Current smoker
20cpd 40yrs
Asbestos. COPD
P18
70yrs. Ex-smoker 1yr
20cpd 60yrs
COPD
P19
RLLapical
L52
Lingula
L54
RUL
L55
MC
L56
RIB
L57
54yrs. Current smoker
30cpd 30yrs
P20
10
20
30
40
50
60
70
80
Time (months)
Normal / squamous metaplasia
Low-grade lesion
High-grade lesion
Invasive carcinoma

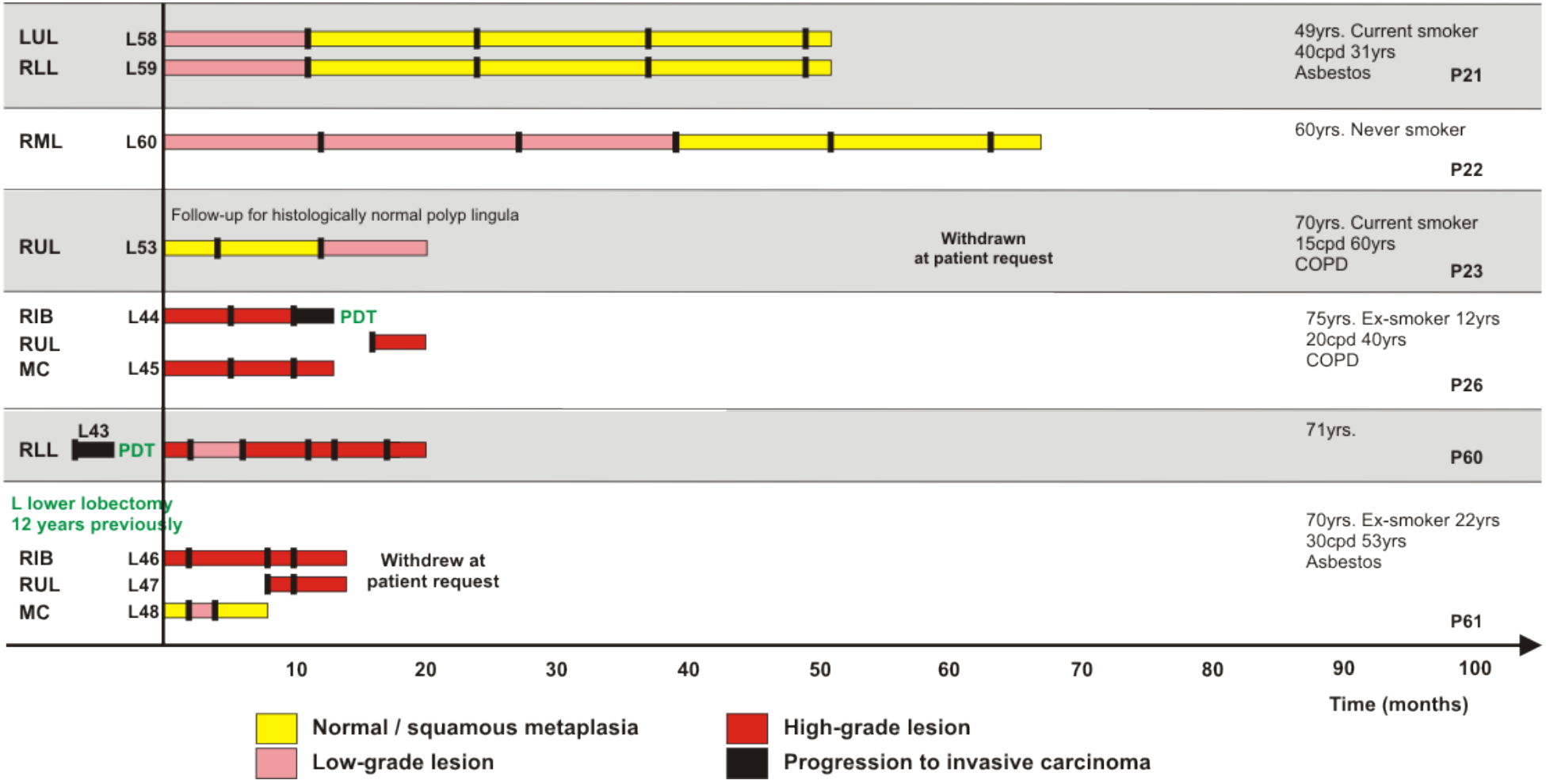
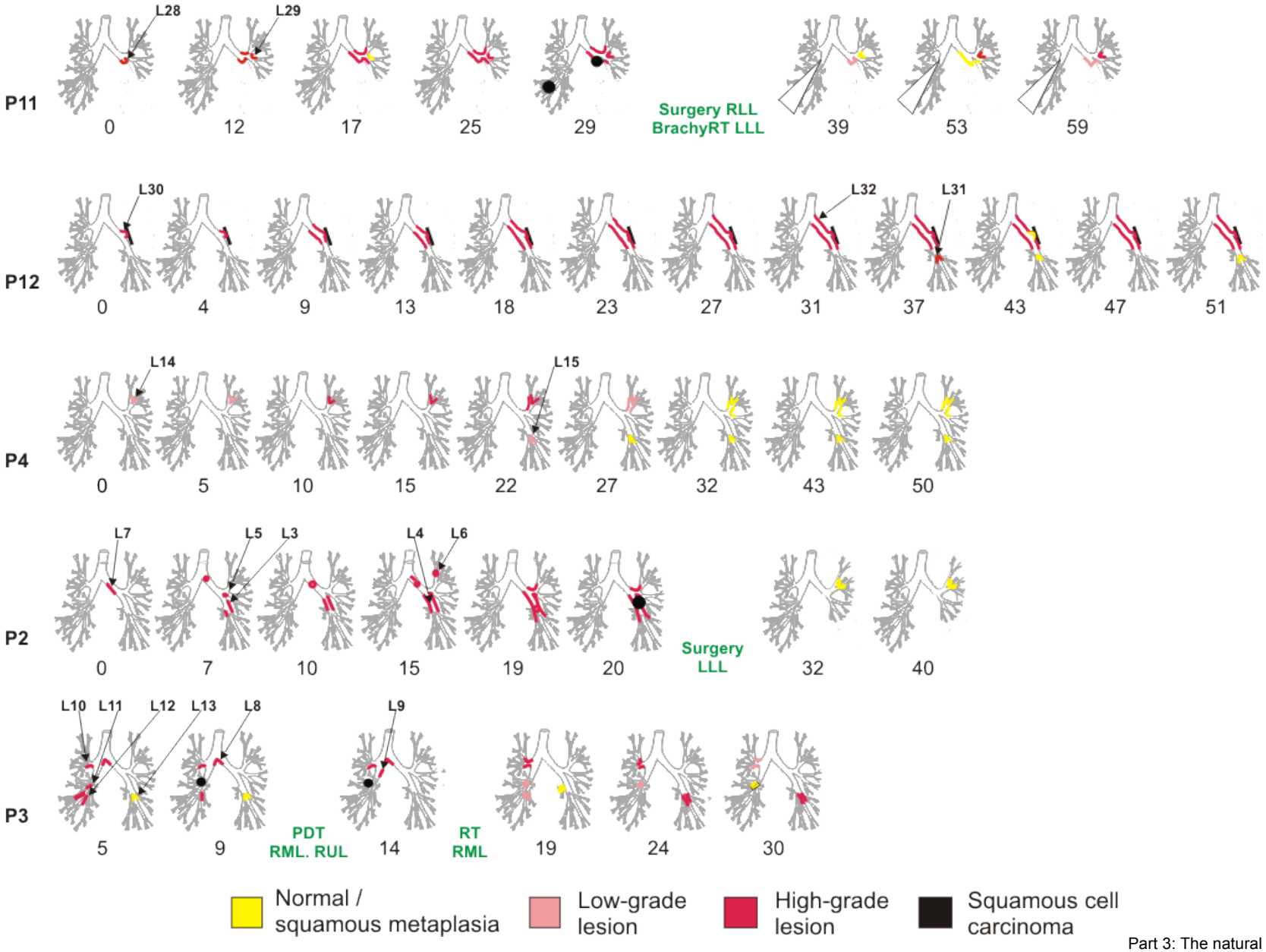

LUL
RLL
L58
L59
49yrs. Current smoker
40cpd 31yrs
Asbestos
P21
RML
L60
60yrs. Never smoker
P22
Follow-up for histologically normal polyp lingula
RUL
L53
Withdrawn
at patient request
70yrs. Current smoker
15cpd 60yrs
COPD
P23
RIB
RUL
MC
L44
L45
PDT
75yrs. Ex-smoker 12yrs
20cpd 40yrs
COPD
P26
L43
RLL
PDT
71yrs.
P60
L lower lobectomy
12 years previously
RIB
RUL
MC
L46
L47
L48
Withdrew at
patient request
70yrs. Ex-smoker 22yrs
30cpd 53yrs
Asbestos
P61
10
20
30
40
50
60
70
80
90
100
Time (months)
Normal / squamous metaplasia
Low-grade lesion
High-grade lesion
Progression to invasive carcinoma

Figure 3.8: The patterns of pre-invasive lesions within individual patients

The bronchoscopic extent and histopathology of lesions are plotted onto a line diagram of the bronchial tree to highlight the temporal and spatial relationships of lesions within an individual patient. The colour of a lesion indicates the histopathology and the key shown on the legend. The length of an abnormality within the bronchial tree indicates the bronchoscopic extent judged by autofluorescence. Lobectomies are shown by erasing the part of the lung that was resected. The L refers to the lesion number used in the text, and the P number refers to the patient number in the UCL study.


P11
L28
L29
0
12
17
25
29
Surgery RLL
BrachyRT LLL
39
53
59
P12
L30
L32
L31
0
4
9
13
18
23
27
31
37
43
47
51
P4
L14
L15
0
5
10
15
22
27
32
43
50
P2
L7
L5
L3
L4
L6
0
7
10
15
19
20
Surgery
LLL
32
40
P3
L10
L11
L12
L13
L8
L9
5
9
PDT
RML. RUL
14
RT
RML
19
24
30
Normal /
squamous metaplasia
Low-grade
lesion
High-grade
lesion
Squamous cell
carcinoma

Observations from the follow-up of patients

Patients develop carcinomas during follow-up

In patients P2 and P3 multiple HGLs were found. New lesions arose during the period of follow-up all of which were high-grade. Only one lesion in each patient progressed to carcinoma, at 25 months in patient P2 and 10 months in patient P3, while the remaining lesions either regressed towards normal epithelium or remained with an unchanged histological diagnosis.

Bronchoscopically some of the lesions increased in size (figure 3.8), while others remained the same size but this was not related to the ultimate outcome of the lesion. Patient P26 had two lesions, L44 and L45, one of which (L44) progressed to carcinoma at 10 months but the other remained histologically and bronchoscopically unchanged. In patient P9 there was an HGL in the left upper lobe which remained stable for 12 months until squamous cell carcinoma developed. In patients P3, P11 and P26, HGLs remained histologically indolent while one lesion within each patient progressed to squamous cell carcinoma.

Two patients developed 2 carcinomas each. In patient P11, the carcinomas were discovered at the same time, one having originated in a HGL that had been unchanged for 30 months, and the other in a remote location in the contra-lateral lung. Patient P1 had two carcinomas, one in the trachea and the other in the right upper lobe. Both appeared to develop directly from HGLs, and both within a short time (6 months) after initial detection.

In 5 patients, carcinoma developed in a location remote from the HGLs under follow-up. In patient P7, lesion L21 remained histologically unchanged while a carcinoma was found distally within the same lobe 12 months after presentation. There was a similar occurrence in patient P6 where a carcinoma was found at month 22 distally within the same segment as the lesion under follow-up. In patient P5 the carcinoma at month 10 was peripherally located in the right mid-zone, unrelated to the 3 HGLs being studied. In patient P10 who had undergone a right pneumonectomy, 3 HGLs were detected at 3 sites (L25, L26 and L27) that

had developed from histologically normal epithelium. None of the HGLs evolved into the invasive carcinoma which was found at month 36 (figure 3.8).

The first detected lesion in patient P4 was LGL in the left upper lobe (L14). At three subsequent bronchoscopies over an 18 month period, HGL was found at the site of the LGL. Following this, LGL and then normal epithelium was identified at that site. A new LGL was detected at month 22 (L15) which appeared to spontaneously regress to normal epithelium. At month 64, some 30 months after the apparent regression of the original lesions, a squamous cell carcinoma was found, but not at the same bronchoscopic location as either of the previous lesions.

Patients with lesions that regressed to normal epithelium

A HGL was the first identified abnormality in patient P8 (L22), who subsequently developed a second HGL in month 14 (L23). Both lesions regressed to normal epithelium, although at the site of lesion L22 there was LGL followed by normal epithelium. At month 32 at the site of lesion L22 there was again LGL, 14 months after the apparent regression of the original lesion.

Lesion regression was seen in patient P13, who had a right middle lobe carcinoma. This was resected by lobectomy, but the resection margin showed a HGL (L33). Two further lesions, both LGL (L34 and L35), were found elsewhere in the bronchial tree at the same time as the carcinoma. All three lesions regressed to normal epithelium, although subsequently at month 18 the site of lesion L33 showed LGL followed by normal histology at the next biopsy. In patient P15, after resection of a left upper lobe carcinoma, there appeared to be regression of a left lower lobe HGL.

HGLs regressed to normal in patients without evidence of other progressive or indolent HGLs, and independent of the risk factor profile of the individual patient e.g. L14 in P4, L22 in P7, L23 in P8, L33 in P13, L37 in P15, L39 in P16 and L52 in P19.

Patients in whom carcinoma was treated with curative intent

Treatment to HGLs applied at the same time as treatment to a nearby carcinoma was not always successful. Lesions L10, L12, L24 and L43 in patients P3, P9 and P60 remained HGL after treatment with photodynamic therapy to invasive carcinomas and the nearby HGLs.

Patients with lesions that remained histologically unchanged

In patient P12, lesions L30 and L32 remained indolent for 58 months and 28 months respectively, whilst regression of lesion L31 was observed. Lesion L30 increased in size significantly over time, eventually involving the left upper lobe stump and the left main bronchus to the orifice of the left lower lobe (figure 3.8). Both of the lesions detected during follow-up in patient P61 remained unchanged histologically. Unfortunately, the patient declined long-term participation in the study.

Patients with multiple lesions

Multiple lesions were found in 18 patients (P1, P2, P3, P4, P5, P6, P8, P10, P11, P12, P13, P16, P17, P18, P20, P21, P26, P61). In 6 patients, progression and regression of lesions was seen within the same patient (P2, P3, P4, P12, P13, P20). There were no specific bronchoscopic or histological features that differentiated lesions that progressed from those that regressed. New HGLs developed in locations bronchoscopically remote from the HGLs under follow-up in patients including P2, P3, P5, P6, P8, P10, P11, P12, P17, P26 and P61. Again, no specific feature in these patients predicted the outcome of individual lesions in terms of regression or progression. The time taken for lesions to evolve varied between patients and between lesions within the individual patient.

The bronchoscopic extent of autofluorescence abnormalities

Information regarding the extent of an individual lesion is limited, as bronchoscopy and biopsy do not permit detailed sampling of the entirety of the lesions under investigation. Therefore the dimensions of an individual lesion were inferred from the autofluorescence bronchoscopic appearances rather than from direct evidence by mapping biopsies. This was

collated with the histopathological findings. The results from patients in whom the dimensions of the abnormal autofluorescence (and by inference the pre-invasive lesions) changed significantly are shown in figure 3.8.

The bronchoscopic extent of some HGLs increased (e.g. L3 in P2, L30 in P12) but remained unchanged in others (e.g. L19 in P6, L28 in P11), although histopathologically the lesions remained at high-grade. The bronchoscopic abnormality in one lesion returned to normal with the regression of the lesion to normal epithelium (L14). There was no association between the bronchoscopic extent of an individual pre-invasive lesion, either at the point of first detection or at any time during follow-up, and the histopathological evolution of the lesion. The rate of change of lesion extent, whether the lesion increased in size or became bronchoscopically smaller did not predict the histological behaviour or outcome of the pre-invasive lesion. The bronchoscopic appearance in terms of colour and nodularity of the lesion (results not shown), either under white light or autofluorescence was not predictive of the histological diagnosis or the natural history of any lesion. The bronchoscopic appearances returned to normal in those lesions that regressed to histologically normal epithelium.

New HGLs arose directly from apparently histologically and bronchoscopically normal epithelium or in the location of a previous LGL e.g. P9. There was no difference in terms of natural history or outcome between newly developed HGLs and those identified at the first bronchoscopy. There was no clear relationship between cigarette smoking and the behaviour or outcome of HGLs.

Discussion

There was bronchoscopic and histological follow-up of 26 patients with 60 pre-invasive lesions of the bronchus for 14-110 months. Pre-invasive lesions progressed to carcinoma, regressed to normal epithelium or remained unchanged for substantial periods of time. Lesions that had appeared to regress to normal epithelium showed pre-invasive lesions at the same bronchoscopic location later in the time-course. Carcinoma developed only in lesions that were high-grade and did not develop from lower-grade lesions and none of the lesions that progressed to carcinoma had regressed at any point in their observed time-course. New lesions were seen to evolve directly from normal epithelium, and developed at any bronchoscopic location at any time, with any natural history and outcome. The autofluorescence findings, both in terms of lesion extent and bronchoscopic appearance, did not predict the underlying histological diagnosis or the histological progress of an individual lesion whether the lesion was of high- or low-grade.

Limitations of the methodology

High-grade pre-invasive lesions are known to have abnormal gap junctions between the cells making sloughing of the mucosa a common occurrence. Biopsy forceps tend to crush bronchial biopsies, and the orientation of the mucosa is often lost due to folding of the sample in the biopsy forceps (figure 4.3). Although internationally accepted guidelines for the diagnosis of pre-invasive lesions are available[8], the classification was based on lesions found in specimens of lung resected for carcinoma. Disruption of the epithelium during bronchoscopic biopsy can cause difficulty in the precise classification of the abnormalities found, particularly between severe dysplasia and carcinoma-in-situ. Within the present study, the biopsies were taken with “cup” forceps using minimal trauma to extract and process the samples. Despite these efforts, biopsies were found to be difficult to interpret due to crush trauma from the biopsy procedure, and epithelial loss in the processing of the biopsy.

To minimise error through possible misclassification of the histological diagnosis of bronchoscopic biopsies, severe dysplasia and carcinoma-in-situ were combined into a single

"high-grade" histology category. Mild and moderate dysplasia were combined into a single "low-grade" histology category. This means that any differences between carcinoma-in-situ, severe dysplasia, mild and moderate dysplasia could not be detected. Interpretation of the histology of small, often crushed biopsies can lead to significant error in the classification of biopsies into these categories, and thus the results of previous studies must be interpreted with caution. Although the differences between the different histological grades were lost in the present study, the accuracy of the histological diagnosis was maintained, improving the reliability of the results obtained.

The evolution of pre-invasive lesions

Progression to invasive carcinoma

The present study followed lesions using serial biopsy, but the progression suggested by previous studies from normal epithelium through pre-invasive lesions of increasing histological and cytological abnormality to the development of carcinoma[106] was not observed. Pre-invasive lesions were noted to progress from low-grade to high-grade histology but the number of lesions in which this was observed was relatively low (5/60, 8%). New lesions were found the majority of which were high-grade at the point of first detection.

Autofluorescence was the main mode of lesion detection used in the present study. It has already been demonstrated that the sensitivity of autofluorescence for low-grade lesions is poor, with as many lesions detected using random bronchial biopsy as were identified using autofluorescence (see Part 2). It is likely that the low detection rate for low-grade histology lesions prior to their development into high-grade lesions is due to the lack of sensitivity of autofluorescence for such lesions.

All of the carcinomas developed from HGLs which agrees with the previous studies that suggested that HGLs carry the highest risk of malignant transformation (Part 1: pre-invasive lesions of the bronchus). Of the 60 pre-invasive lesions under observation, 46 showed high-grade histology during the course of the study, but carcinoma developed in only eight lesions during this period of follow-up. Most of the previous studies classified lesions by their initial

histology and consequently may have pre-selected lesions at the highest risk for malignant change, which may not be representative of all lesions attaining high-grade histology (see Part 1: pre-invasive lesions of the bronchus). In the present study follow-up was longer than most of the previous studies but was not of sufficient duration to establish whether all the HGLs would progress to malignancy. There were no specific clinical, radiological or bronchoscopic features that predicted the risk of progression to malignancy for an individual lesion.

HGLs may continue to grow and progress across the epithelium, with the bronchoscopic as well as the histological extent of the lesion increasing over time. This is not clearly associated with invasion, although it is difficult to determine the presence of invasion within such a large area of abnormality (e.g. lesion L3, in whom a small focus of invasion was found within a large area of CIS[117]). At present, even when there is a large HGL in the bronchial tree, neither the risk of invasion nor the specific focus of invasion can be determined with any reliability. Further studies are required to identify specific bronchoscopic or biological features that can determine the presence of invasion within any given lesion.

Regression to normal epithelium

Regression of lesions to histologically normal epithelium was observed in some lesions. The mechanisms by which regression occurs are not known. Pre-invasive lesions are thought to accumulate genetic damage during their evolution due to the effects of exposure to carcinogen[118]. It is possible that as a lesion evolves genetic damage, amplification of parts of the genome and loss of function of others may lead to gene rearrangements that may be lethal, or activate apoptosis, rather than confer a growth advantage. Alternatively, the lesion may fail to develop a blood supply through failure of angiogenesis due to a lack of, or an inability to produce, appropriate growth factors or signalling mediators and so may not be able to grow or develop further, leading to involution of the lesion. Further work is required to define the mechanisms by which regression of lesions occurs, using genome and RNA analysis techniques, and also examination of the tissue interactions between pre-invasive lesions and their local environment.

Some observations call into question whether histological regression of pre-invasive lesions genuinely occurs. At the bronchoscopic site of lesions of both high and low grade that were deemed to have regressed, pre-invasive lesions were found in later biopsies (e.g. L22, L33, L54, L57). There is also the finding that serial sampling of a lesion shows biopsy to biopsy variation in the histopathological diagnosis. Lesions such as L19, L29 and L30 showed high-grade histology interposed between normal histology. There may be several explanations for this.

1. There may be failure to accurately re-biopsy the lesion. Once biopsied, a bronchoscopic location has abnormal autofluorescence for a number of months, making re-biopsy of the exact location straightforward[26]. Careful mapping and recording of the location of biopsied abnormalities was undertaken to further minimise this potential source of error.

2. It could be suggested that the technique of biopsy debulks the lesion and that this accounts for the histopathological changes observed in pre-invasive lesions[26]. This technique disrupts the lesions, and the effect of this disruption has not been adequately studied and may have several consequences.

    a. The lesion may take a variable amount of time to regrow depending on the biology of the lesion. Furthermore, it is possible that epithelial healing after the trauma of biopsy may cover the originally sampled lesion, which takes time to penetrate the relatively normal overlying healing epithelium. This theory is less plausible as pre-invasive lesions usually have a growth advantage over histologically normal adjacent epithelium, but support for this idea may be found in lesions L2, L19, L24, L29 and L30 where a series of abnormal biopsies is punctuated by histologically normal samples.
    b. Disruption of lesions using photodynamic therapy has been shown to affect their outcome through an immunological effect[119,120], and it is possible that disruption of small pre-invasive lesions due to biopsy may have a similar effect and consequently

influence their natural history. There are techniques in development that permit analysis of lesions in vivo without their physical disruption, including optical biopsy[121], high-resolution bronchoscopy and confocal microscopy[122]. When these techniques are as reliable, well studied and understood as biopsy and histological analysis, studies of the natural history of observed pre-invasive lesions should be repeated, using a non-disruptive method of sampling.

c. It has been suggested that biopsy may completely remove the lesion[26]. When the dimensions of the pre-invasive lesions in the study were assessed under autofluorescence they were found to be larger than could be removed at biopsy. This argues against the total removal of lesions by biopsy as a mechanism for the change in the histology at the bronchoscopic locations under observation.

3. The lesion may indeed have regressed and cells from a different lesion or a new clone of cells may have grown or migrated into the bronchoscopic epithelial site previously occupied by the pre-invasive lesion.

These theories cannot be explored using conventional histopathological techniques, as they are of insufficient sensitivity to differentiate between lesions that have the same histological grade, but different progenitor cells. There is some evidence to suggest that cells migrate along the bronchial epithelium[123]. This is supported by evidence of similarities between the genetic profiles of bronchoscopically remote lesions[110,124] but against this is the finding of discordant genetic profiles in multiple lesions in remote locations within the bronchial tree when sampled at a single time-point[110]. These data suggest that the currently accepted definition of regression, i.e. “the presence of a histologically lower grade of pre-invasive lesion than the presenting lesion in two consecutive biopsies”[26,27] may not be appropriate and that further work in this area is necessary.

The above findings have implications for future research, both in terms of the evaluation of the natural history of pre-invasive lesions, but also the outcome of therapies. A series of

biopsies, rather than a single set of biopsies taken at a single time-point, are required to fully evaluate the long-term behaviour of the bronchial epithelium, or the effect of an intervention on the bronchial epithelium. Genetic studies of cells and lesions to determine their clonal relationships both across the whole epithelium at a single time-point, and also at a single bronchoscopic location over a period of time, are required to clarify these issues.

Deficiencies of the histopathological classification

The internationally accepted WHO classification of pre-invasive lesions implies that pre-invasive lesions within each histological grade are identical and consequently there is no subdivision or subtyping[8]. The present data suggests that pre-invasive lesions of the same histological grade can have radically different natural histories and outcomes. The study by Pasic et al.[44] in which resection margin CIS was followed with serial bronchoscopy is of particular relevance. They found that CIS could be subdivided depending on the histopathological features and identified CIS with extension into submucosal gland acini, CIS extending into submucosal gland ducts and CIS involving surface epithelium only. Each of these subtypes had different outcomes in terms of progression to malignancy. Keith et al.[41] described the phenomenon of "angiogenic squamous dysplasia", and showed differences in the behaviour of lesions carrying this abnormality compared to standard dysplasia. An older Japanese study[125] described "nodular" and "superficial spreading" types of CIS, and suggested that these histopathological morphologies had different outcomes. These data suggest that the current classification of pre-invasive lesions[8] may be too crude to differentiate lesions that will progress to malignancy from those that will not. Further study of high-grade pre-invasive lesions with particular reference to histopathological features including cellular and histological ultrastructural features, depth of invasion into glands and the size and extent of lesions may be of relevance in the determination and prediction of the evolution of a given individual pre-invasive lesion.

Implications for the clinical management of lesions and interpretation of studies of treatment

Currently the histological diagnosis accorded to an individual pre-invasive lesion when first identified is used as the baseline from which the subsequent natural history of the lesion is

plotted[8]. This means that if the lesion is an LGL at diagnosis, and HGL at later biopsy, it would be considered to have progressed[26,27,39]. The variability observed in the histology of pre-invasive lesions over time means that the baseline histological diagnosis accorded to an individual pre-invasive lesion based on the initial biopsy may not accurately reflect the histology of the lesion over the preceding months.

Biopsies at a single time-point are markers along a journey for the individual lesion, which can change direction at any point, with some lesions progressing and then regressing, and others continuing to progress. The labelling of a lesion based on a single biopsy or 2 biopsies a few months apart can lead to errors in the prediction of lesion behaviour and outcome. For instance, the biopsy taken from lesion L21 at month 10 might suggest a low-grade lesion that subsequently progressed to a high-grade histology, whereas review of the entire sequence of biopsies showed that the lesion was essentially at high-grade with a single episode of low-grade histology. Similarly, for lesion L14 the month 8 apparent progression from low-grade to high-grade was then followed by histological regression to normal epithelium but not for another year. The subsequent evolution and behaviour of any lesion and the highest histological grade attained by an individual lesion cannot be predicted from a single biopsy, or in many cases two consecutive biopsies a few months apart. This has implications for the clinical management of pre-invasive lesions and the interpretation of previous shorter-term studies of the natural history of pre-invasive lesions.

The objective of any management strategy is to identify and successfully treat the lesions at highest risk of progression to invasive carcinoma. No bronchoscopic, histological or clinical factors that predict the outcome of pre-invasive lesions could be identified using the data collected. This suggests that the natural history of an individual lesion is unique to the lesion, rather than patient-based or dependent on currently identifiable patient factors. Lesions attaining high-grade histology are at greater risk of developing malignancy than any other histology, but the risk remains relatively low for an individual lesion. During the present study, carcinoma developed in 11 of the 26 patients, but in only 8 of the 46 lesions attaining high-grade at some point in their observed natural history. The presence of HGLs predicted

a higher risk of carcinoma development, not only within the lesion under observation, but also at remote locations within the lungs and bronchial tree.

The present study suggests that the outcome of a lesion cannot be predicted by the initial histology or from short-term follow-up unless the lesion reaches a critical outcome, i.e. progression to fully invasive carcinoma. The development of new lesions at any location in the bronchial tree at any time-point suggests that patients harbouring these types of lesion require long-term follow-up of the entire visible bronchial tree using autofluorescence bronchoscopy, and the non-visible bronchial tree and lung parenchyma using CT scanning. High-grade pre-invasive lesions may be a marker for later carcinoma development, the location of which cannot be predicted using currently available parameters.

Novel markers that establish the malignant potential of individual manifest lesions are needed. These might include molecular biology, immunohistochemical and proteomic markers. Although it is possible that a single marker will identify lesions that require treatment, it is more likely that a panel of markers will be used to assess the risk of malignancy for any given lesion. In parallel, markers to assess the response to treatment, and in particular a reduction in the malignant potential of treated lesions, must be developed as the natural history of a given lesion is uncertain. Previous studies of endobronchial therapies to pre-invasive lesions are difficult to interpret as the untreated natural history and the malignant potential of treated lesions were not known (see section 1: pre-invasive lesions).

Summary

The objective of this study was to confirm that squamous cell carcinoma of the bronchus develops from carcinogen-exposed bronchial epithelium through a series of pre-invasive lesions of increasing histological and cytological abnormality, determine the time-course of pre-invasive lesion development and determine whether pre-invasive lesions are committed to the development of malignancy. The progression of carcinogen-exposed bronchial epithelium to squamous cell carcinoma through a series of pre-invasive lesion was not seen.

HGLs were seen to develop into malignancy, but not all lesions were committed to the development of carcinoma, with 8 out of 46 lesions exhibiting such progression.

The natural history of pre-invasive lesions is variable with an unpredictable time-course. Some lesions progress, some regress and some remain unchanged histologically. Different lesions in a single patient may have different natural histories and different outcomes. Short-term follow-up may misrepresent the long-term evolution of an individual lesion or bronchoscopic location. Consequently decisions regarding the clinical management of pre-invasive lesions cannot be determined solely on the basis of a single biopsy, or a short-term period of follow-up. Further work is required to identify novel markers of malignant potential, and response to treatment.

The results of the present study are affected by selection bias, as only patients with symptoms or clinical suspicion of lung cancer underwent initial evaluation. It is not clear whether lesions in these patients are unique, or whether lesions in asymptomatic patients behave similarly. The current literature does not provide sufficient information to enable the development of effective management guidelines or interpret studies of therapy. Much work remains, and given the difficulties in finding lesions for study, multi-centre collaboration may be necessary.

The results of genetic studies of biopsies from the present study should determine the clonal relationships between the lesions under observation. If the biopsies which are histologically more normal show a different genetic profile from the original lesion then this supports the theory that another clone of cells grew into the biopsy site and grew over the original lesion, which subsequently regrew and became manifest on later biopsies. It is interesting that biopsies from some of the bronchoscopic locations in which pre-invasive lesions appeared to have regressed showed another abnormality at a later time. The relationship of these lesions to the original pre-invasive lesion is not clear, but the mechanism by which a lesion of higher-grade histology can evolve into one of a lower grade is not immediately apparent. It is more likely that the later lesion originated from a different clone of cells and was therefore

unrelated to the original pre-invasive lesion. Comparison of the genetic profiles of the earlier and later lesions, should determine their clonal relationships and would provide important insights into the natural history of carcinogen-exposed bronchial epithelium, and the relationships between manifest lesions.

Part 4: The molecular biology of pre-invasive lesions of the bronchus

Hypothesis

The pattern of genetic loss within lesion cells drives the evolution and determines the outcome of individual pre-invasive lesions of the bronchus.

Objective

Investigate the relationship between loss of genetic material at specific chromosomal loci and the behaviour and outcome of pre-invasive lesions of the bronchus. Identify consistent patterns of genetic loss associated with the behaviour and outcome of lesions.

Method

The study protocol was approved by the University College London / University College London Hospitals committees on the ethics of research on human subjects study no 01/0148. Written informed consent to enter the study was obtained from each participant prior to study entry.

Patient selection

Patients were selected from those in the "Natural history of pre-invasive lesions of the bronchus" study during which biopsies were obtained from lesions followed bronchoscopically. In the study, biopsies were collected into formalin for processing into blocks for DNA extraction and also fresh frozen into OCT gel and dry ice for later RNA extraction.

Selection criteria for the molecular studies:

1. Patients with pre-invasive lesions of the bronchus followed within the "natural history of pre-invasive lesions of the bronchus" study.
2. Samples available for DNA extraction for analysis.

The 5 patients in the study were chosen after analysis of the natural history diagrams in "the natural history of pre-invasive lesions of the bronchus" study. All of the patients selected had lesions with a clear sequence of changes i.e. lesions that progressed to invasive carcinoma or lesions that regressed to normal epithelium, and sufficient samples at each time-point for analysis.

Patient P6 has one HGL and another HGL that progressed to squamous cell carcinoma.

Patient P8 had two HGLs that regressed to normal epithelium.

Patient P12 had a HGL at a bronchial resection margin that progressed to involve an extensive area from the left main bronchus to the orifice of the left lower lobe bronchus.

Patient P4 had a HGL and a LGL that regressed to normal epithelium.

Patient P11 had a previous carcinoma that was resected, a HGL that developed into squamous cell carcinoma and a further synchronous squamous cell carcinoma in the contralateral lung.

Sample selection

The samples for microdissection and DNA extraction were selected by analysis of the results of the "Natural history of pre-invasive lesions of the bronchus" study and cross-referencing with the database of collected samples. A diagrammatic representation of the bronchial tree was used to highlight this (figure 4.1).

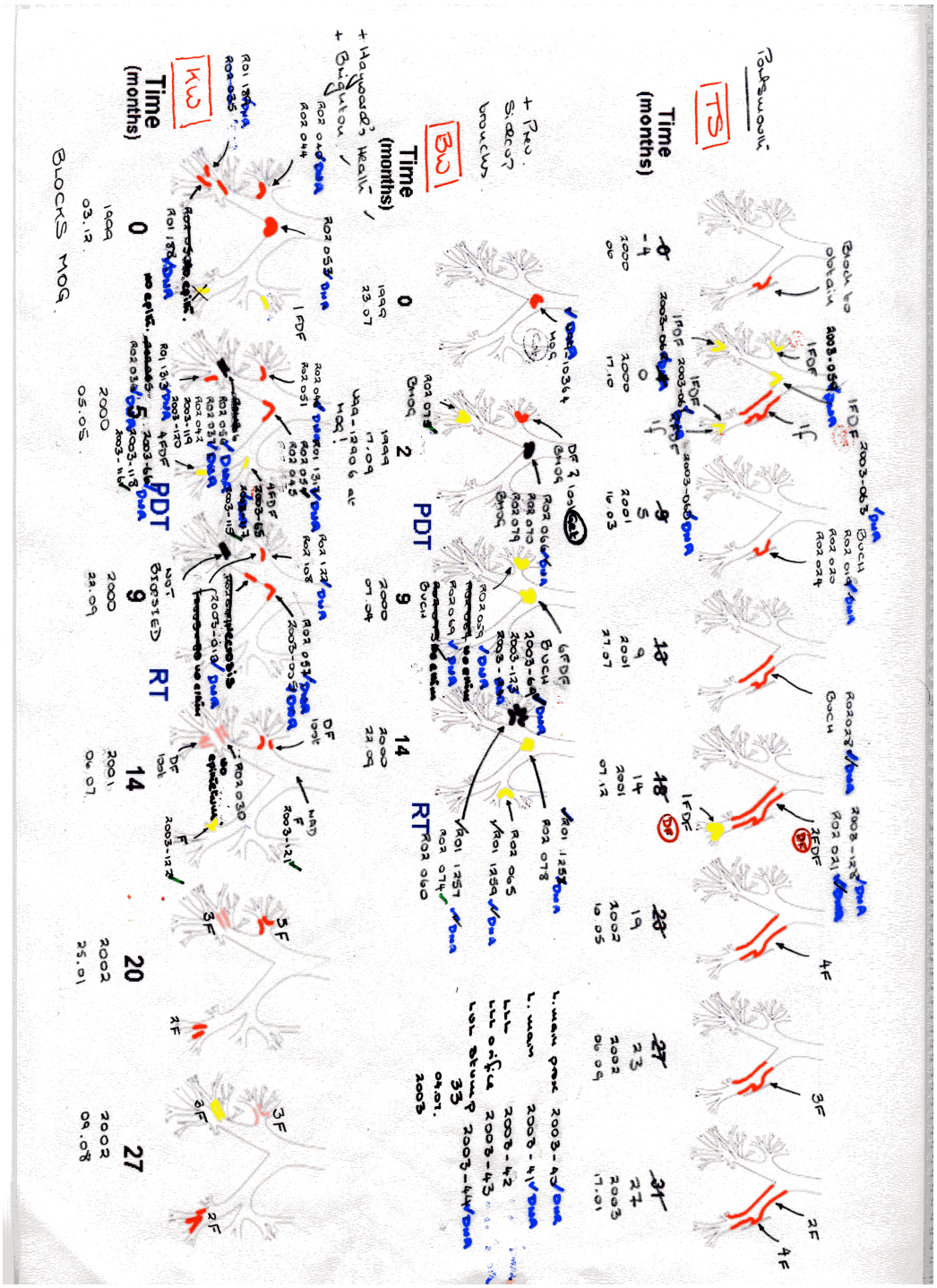


Figure 4.1: A diagram showing the results of bronchoscopies and their relationship to the samples collected and DNA extracted. The numbers e.g. 2003-63 and R02 028 refer to the blocks containing the bronchial biopsies from the bronchoscopic location identified. The bronchoscopic findings are shown on the representative bronchial tree diagrams, with the colour indicating the histology at that location at that time-point.

Biopsy processing for DNA extraction

1. Formalin-fixed specimens

Placed onto acetate strips and immediately fixed in at least 10 times the sample volume of 10% neutral buffered formalin for 4 hours to ensure fixation but minimise exposure of the biopsy to formalin. Formalin fixes specimens by producing protein cross-links and excessive fixation times have been found to inhibit DNA extraction. The biopsies were processed into paraffin wax blocks using a Leica™ TP1050 Vacuum Tissue Processor. This uses an overnight cycle involving dehydration of the biopsy in 70%, 90% and 100% ethanol and clearing in xylene. The fixed biopsy was then blocked into paraffin wax using the Blockmaster™ system supplied by Raymond A. Lamb™ using the manufacturers protocol.

2. Frozen specimens

The specimen vial containing the biopsy frozen in OCT gel was removed from the –80°C freezer and allowed to defrost completely. This was confirmed by the OCT returning to a clear gel from an opaque white state when frozen. The biopsy was lifted out of the OCT gel with the minimum of trauma using a fine 25G needle and placed directly into at least 10x the biopsy volume of 10% neutral buffered formalin. After 4 hours the biopsy was transferred into 70% ethanol for transportation to the histopathology department for processing into a paraffin wax block using the protocol for formalin-fixed specimens above.

<u>Preparation of sections for Laser Capture Microdissection</u>

The paraffin was block frozen to -4°C and trimmed so that 4-5 sections in a single ribbon could be fitted onto a single microscope slide. Ribbons of 4-5 7μm sections were cut using a Shandon AS325 Retraction Rotary Microtome and placed onto the surface of a water bath at 37°C. The sections were transferred on to standard uncoated glass microscope slides without adhesive. The slide and the sections were blotted with filter paper and left to air dry at room temperature. This minimised the adhesion of the section to the slide and allowed optimal microdissection. The block was cut until a minimum of 10 sections were obtained form the centre of the biopsy. 5μm sections were found to provide clear cellular and lesion definition when stained for both diagnosis and for laser capture microdissection with the

Arcturus Laser Capture Microdissection system. However, the 5μm sections were found to be too adherent to the slide for reliable microdissection. 7μm sections were less adherent and dissection from the slide was straightforward. At 7μm section thickness the cellular and lesion morphology was less well defined, but was sufficient to facilitate microdissection (figure 4.2).

Staining for diagnosis and lesion identification

One slide from near the centre of each biopsy containing 3-5 sections was hand-stained with haematoxylin and eosin using a standard protocol. Vectamount™ (Vector™ Laboratories, UK) was used to mount the coverslip. A representative haematoxylin and eosin stained section was photographed using the Arcturus Laser Capture microdissection microscope with the visualiser arm in place. Multiple overlapping photographs through the x10 objective were taken and were assembled to give a large high magnification picture using Corel Photopaint software on the Macintosh OSX and printed onto Kodak everyday picture paper using an Epson Stylus Photo 830 5600dpi colour inkjet printer for maximum resolution (figure 4.3). The diagnostic slide was reviewed with a Consultant Histopathologist with a special interest in pulmonary malignancy. The epithelial lesion was identified and mapped onto the photomicrograph of the section (figure 4.3).

Figure 4.2: Photomicrographs of example sections. At 5μm thickness (top sections) there is clear lesion and cellular definition. At 7μm (lower sections) the lesion and cellular definition is less clear.

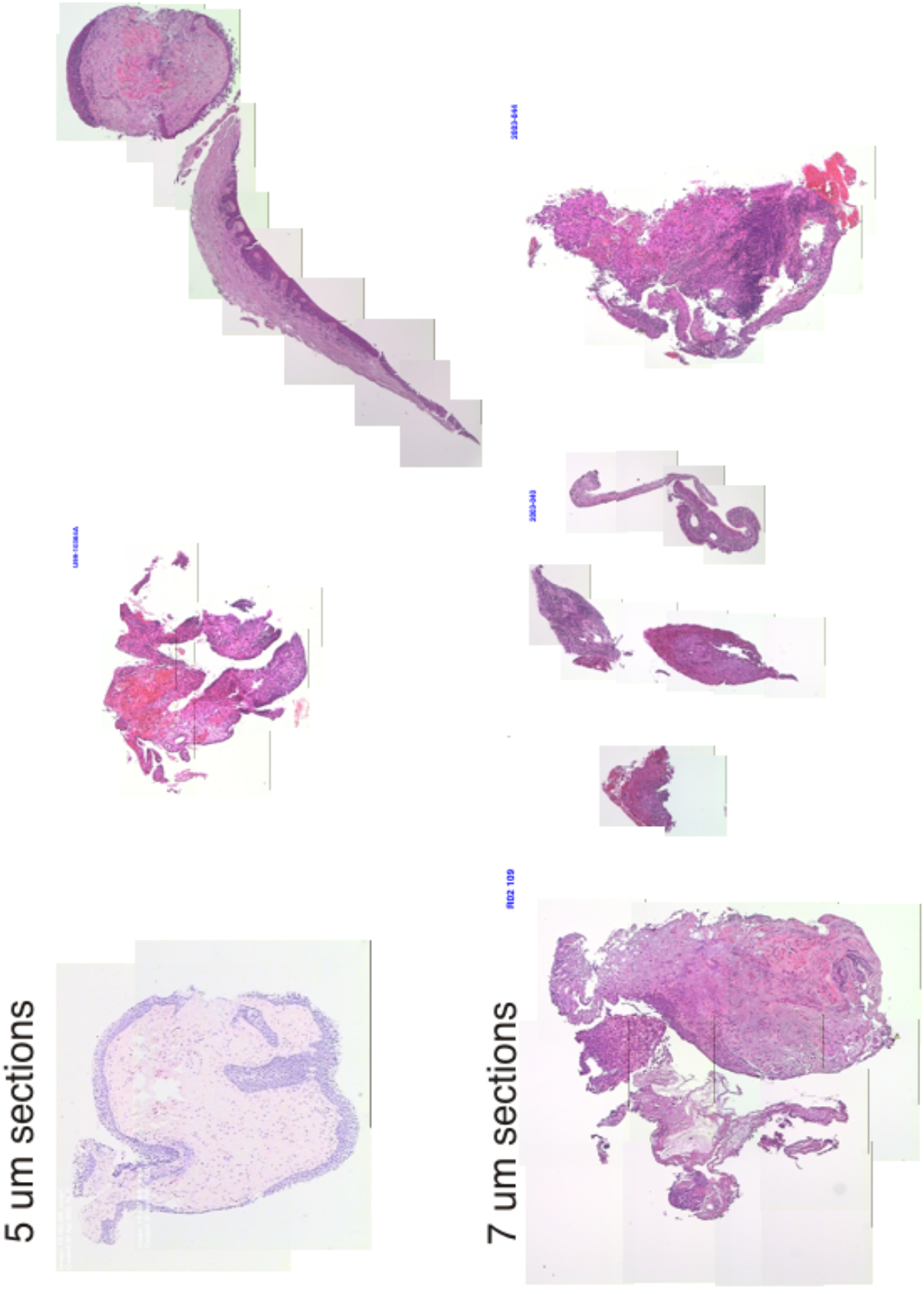
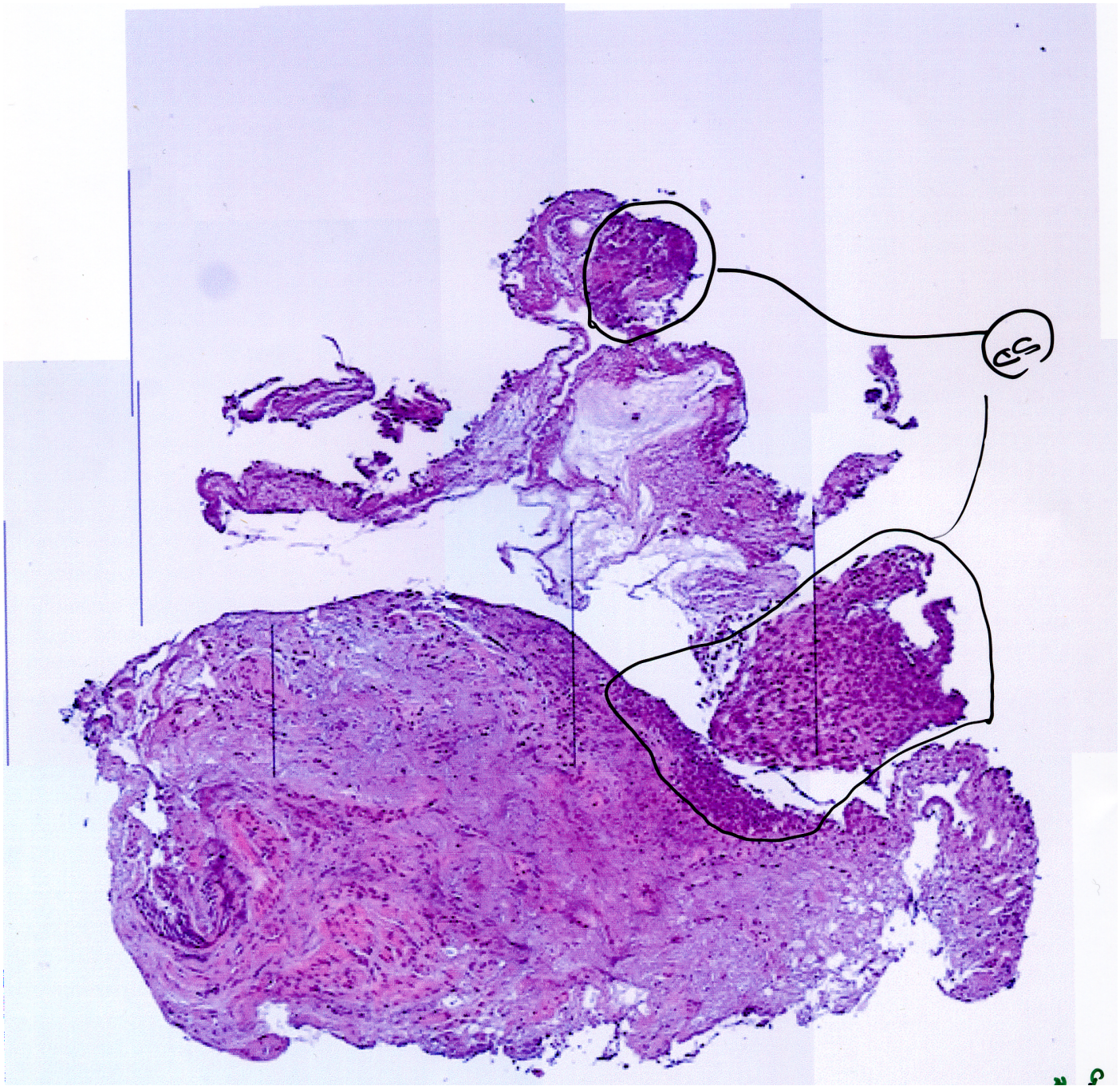


Figure 4.3: An example section with the abnormalities highlighted. The areas with “SD” (severe dysplasia) are shown.



Laser Capture Microdissection

A series of experiments were designed to optimise each part of the laser capture microdissection and DNA extraction protocol and are presented in summary form.

1. The staining method used to define the cells in the section and facilitate microdissection.
2. The power of the laser used for microdissection.
3. The digestion buffer used for DNA extraction.
4. The length of time for DNA extraction from the microdissected cells.
5. Quantification of the extracted DNA.
6. Dilution of the extracted DNA for LOH analysis.

Laser Capture Microdissection was performed using the Arcturus Laser Capture dissection microscope. The lesion of interest was microdissected using the photomicrograph map and the Histopathologists directions as a guide. The Laser Capture Microdissection from each biopsy was performed using as many slides and sections from the original biopsy as necessary to provide at least 50% coverage of the Laser Capture Microdissection cap. For carcinoma this could be fulfilled using a single section, but for pre-invasive lesion and normal epithelium biopsies, this typically required 10-15 sections. The objective was to provide the greatest possible quantity of DNA, and approximately equivalent quantities of DNA from each bronchoscopic location.

1. Slide Staining Protocol for laser capture microdissection

There is evidence that staining with haematoxylin may affect PCR on microdissected DNA. Nuclear Fast Red is thought to avoid this issue, but may give less effective definition of the lesion cells for microdissection. Haematoxylin using a short (1 second) dip protocol was found to give DNA of quality similar to that of Nuclear Fast Red stained specimens when compared during microdissection of a single lesion. Consequently a short exposure to haematoxylin was used (slide dipped in haematoxylin) to define the lesion of interest prior to microdissection.

2. Laser Power during microdissection

It has been suggested that a higher laser power affects a larger area of the cap during microdissection, but may cause damage to the specimen, thus reducing DNA yield. The yield of DNA using a 50W and 100W laser power was compared. The cap was more effectively melted with a 100W laser power; at 50W areas of the glue did not melt adequately and specimen was left on the slide in a patchy distribution risking loss of specimen. At 100W this was eliminated, with no reduction in DNA quality for PCR.

3. Digestion buffer for DNA extraction from microdissected cells

Two different buffers were assessed for use in the digestion of microdissected cells. There was no difference between the different buffers used. Proteinase K and Tween 20 digestion buffer was selected due to familiarity with the buffer within the laboratory.

4. Duration of digestion of the microdissected specimen

Overnight digestion of the microdissected specimen was found to produce the most reliable clearance of the microdissection cap and therefore adequate DNA extraction for PCR analysis.

5. Quantification of the Digested DNA product

It was found that both proteinase K and Tween 20, used in the protocol for digestion of the Laser Capture dissected specimens gave a positive result on picogreen assay. This invalidated the results, and made quantification of the DNA impossible. Purification of the DNA was not attempted as there was very little DNA from each specimen due to the small size of the lesions under investigation, and it was decided not to risk loss of DNA in processing when the PCR protocols were known to work with unpurified samples.

6. Dilution of the DNA product for LOH analysis by PCR

A series of dilutions of the template extracted DNA was used to determine the optimum dilution for PCR. Dilutions of 1:20, 1:10, 1:5 and no dilution were used. The most consistent

and best quality results were obtained with 1:5 dilution. At 1:10 and 1:20 dilutions there were some failures of PCR. 1:5 dilution of the DNA product was selected for the studies.

Loss of Heterozygosity analysis

Selection of method

Loss of heterozygosity analysis was selected as:

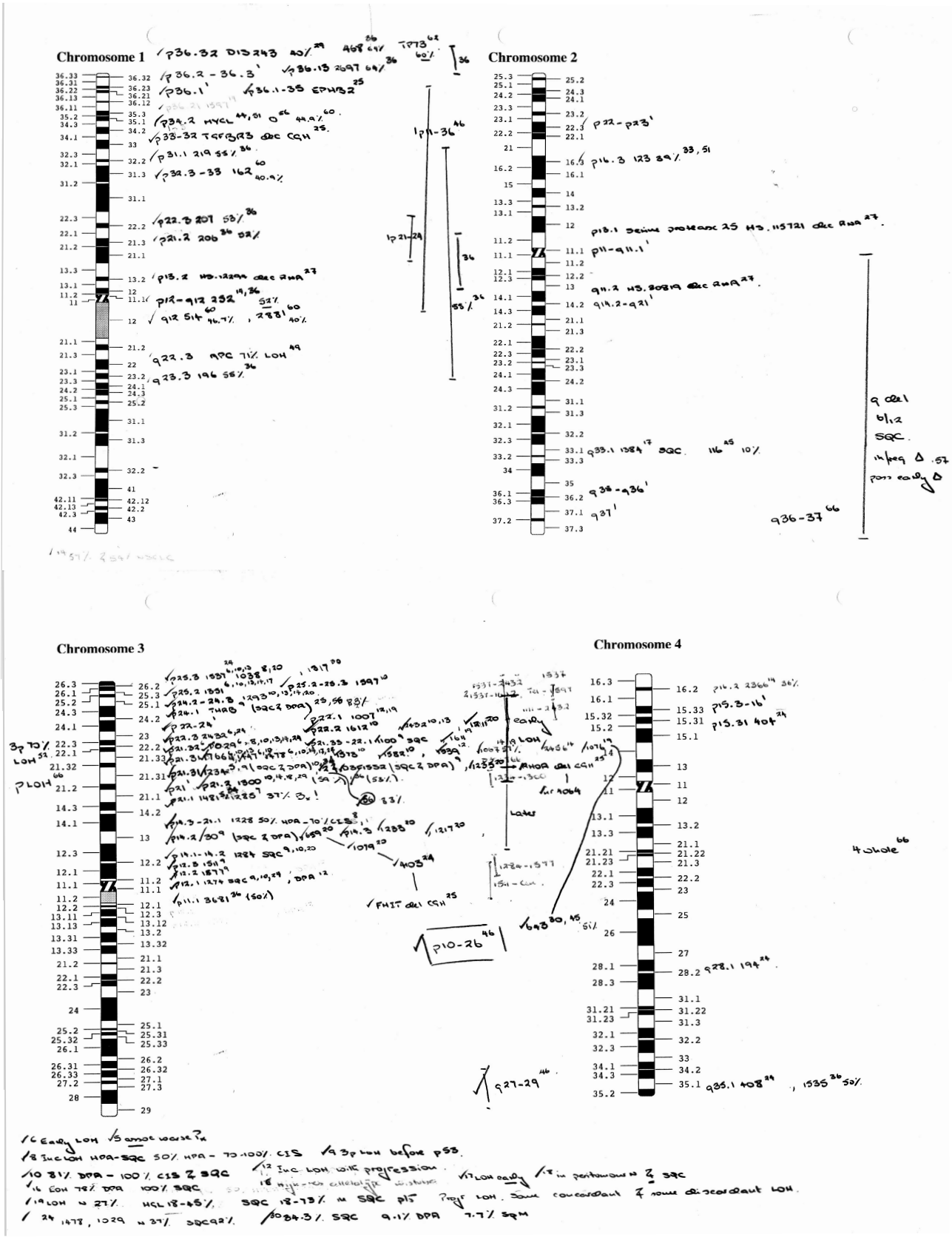
i. This technique has been used extensively and successfully to detect genetic loss in a wide range of carcinomas including lung.
ii. In a previous pilot study of one patient[117], differences in the loss of heterozygosity pattern was found between pre-invasive carcinoma-in-situ and the post-invasion squamous cell carcinoma that developed in the same anatomic location.
iii. Detailed allelotyping is possible using this technique with a better resolution for genetic loss than is possible with whole genome methods such as comparative genome hybridisation.
iv. The DNA extracted from the bronchial biopsies is optimised for PCR-based analysis.
v. Loss of heterozygosity has been extensively studied in squamous cell carcinoma of the bronchus but there are few data from studies of pre-invasive lesions. However, it is suggested that genetic loss occurs in a sequential manner during the progression of pre-invasive lesions to carcinoma of the bronchus, and therefore studies of genetic loss and their relationship to the behaviour of pre-invasive lesions are required.

Selection of chromosomal loci for analysis

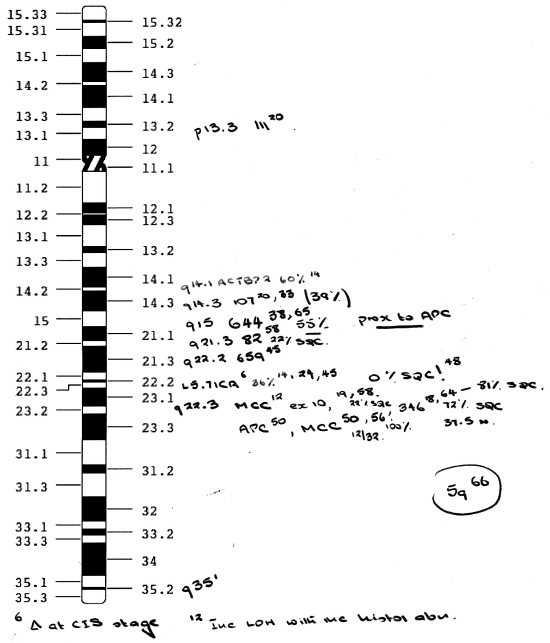
Previous studies of genetic loss in squamous cell carcinoma of the bronchus were found by a search of the Pubmed database and reference lists from the papers obtained from Pubmed. Chromosomal locations and polymorphic microsatellite markers that showed genetic loss and the frequency with which it was found within the specimens analysed was recorded where available. The majority of the studies were 5-10 years old and so the markers used in the studies were remapped onto the latest draft of the human genome (2003, using the NCBI UniSTS and the Genome Database) and plotted onto an ideogram (figure 4.4). This approach revealed candidate locations in the genome where genetic loss

appeared to be a frequent occurrence in squamous cell carcinoma of the bronchus. This information was used to determine which chromosomal locations would be analysed in the pre-invasive lesions. The chromosomal locations selected were: 3p (3p12, 3p14, 3p21 and 3p25), 4p16, 5q (5q15 and 5q21), 8p22, 9p16 and 17p13. Some loci, such as 3p, 9p and 17p had been shown in previous studies to be associated with early stage pre-invasive lesions and squamous cell carcinoma of the bronchus. Other loci, such as 5q15 and 5q22 were chosen as known tumour suppressor genes had been found in the loci, and there had been consistent LOH found in previous studies of squamous cell carcinomas (figure 4.4). 4p16 was selected as previous work in the laboratory had shown an association between LOH and the progression of an HGL to squamous cell carcinoma in one patient.

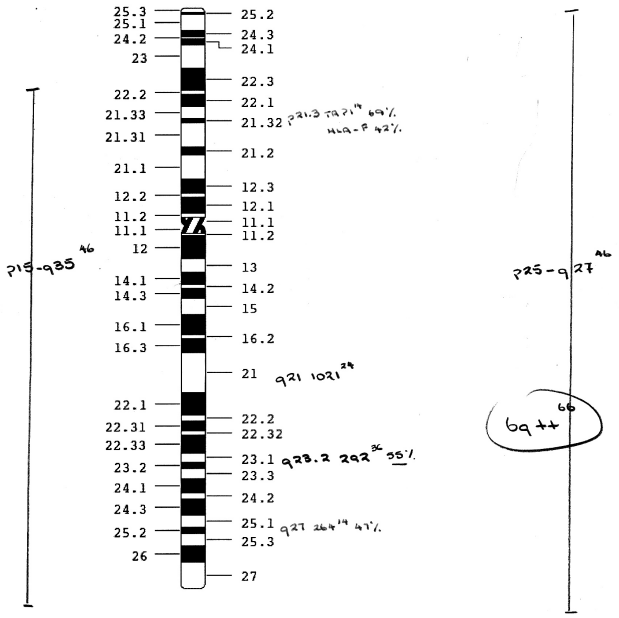
Figure 4.4: Ideograms showing genetic loss throughout the genome in squamous cell carcinomas. The reference numbers do not correspond to the reference numbers within the reference list. The percentage of squamous cell carcinomas showing loss are shown where the data were available.



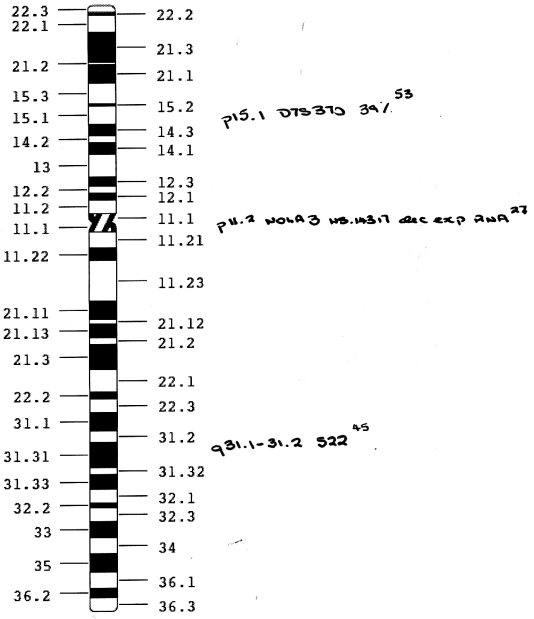
Chromosome 5



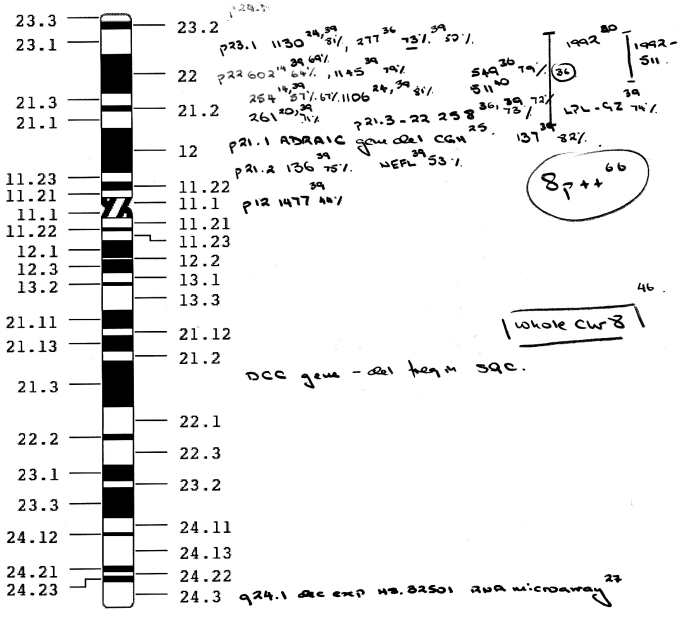
Chromosome 6

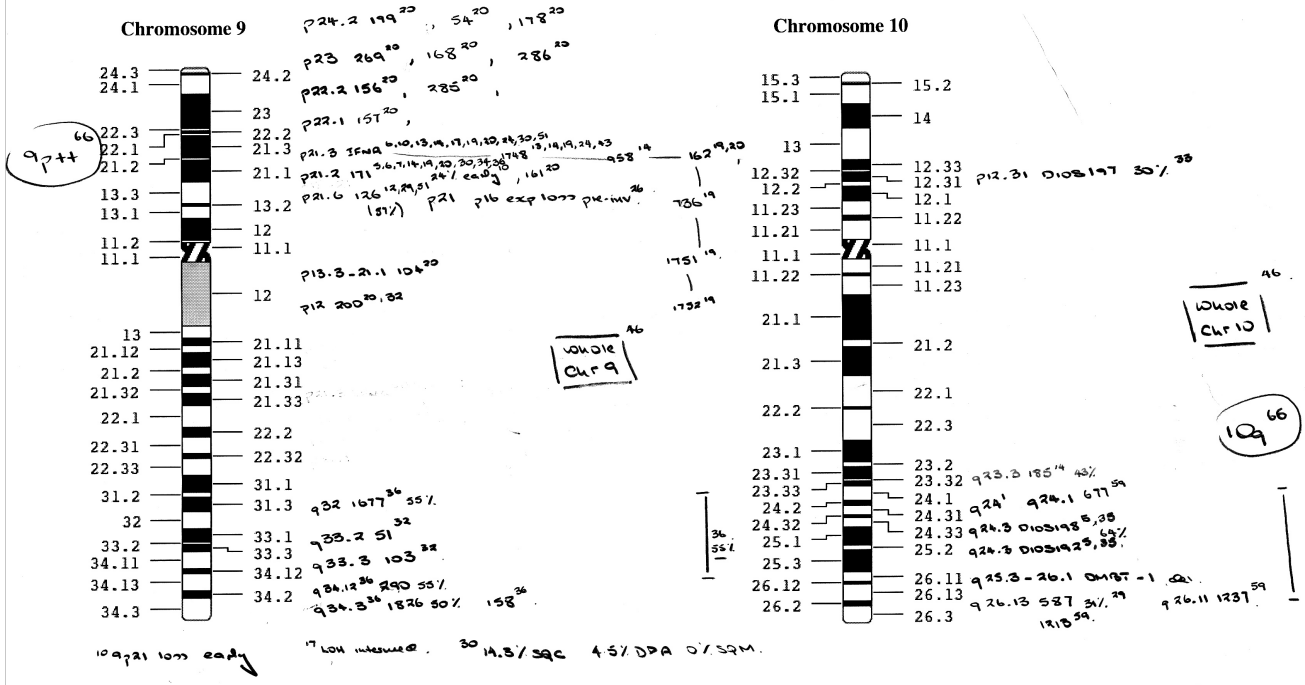


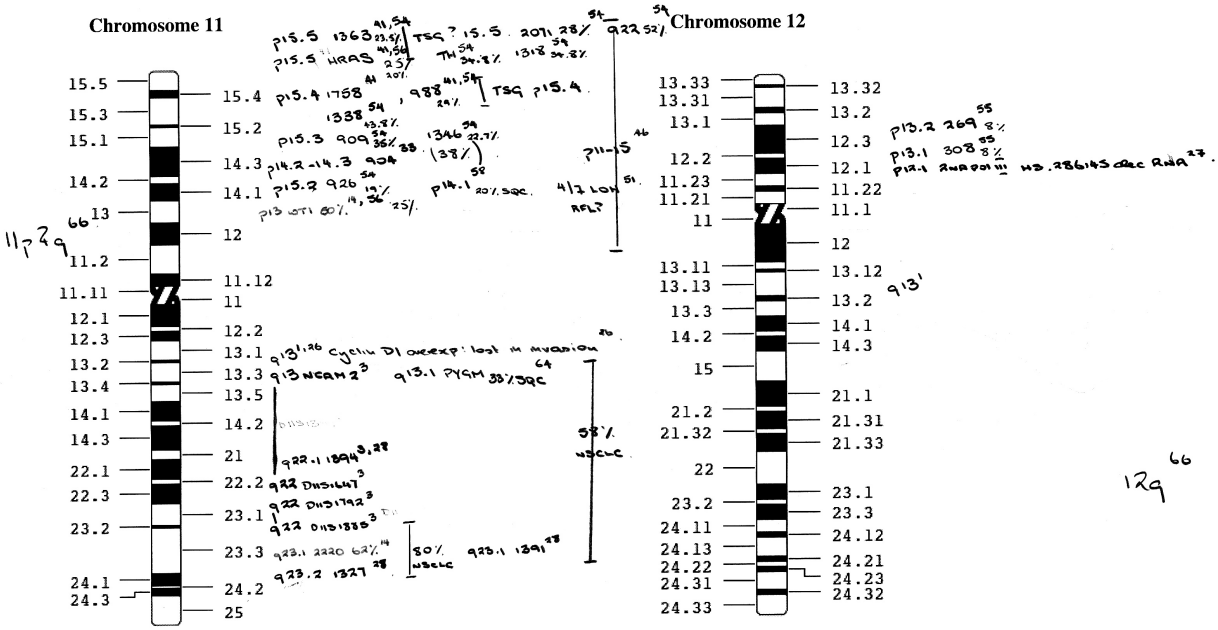
Chromosome 7



Chromosome 8

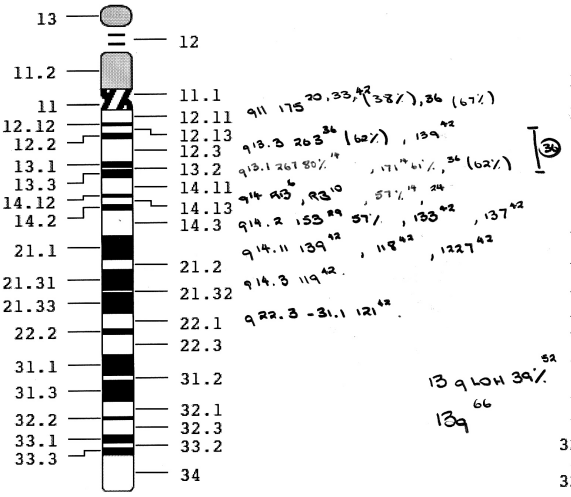




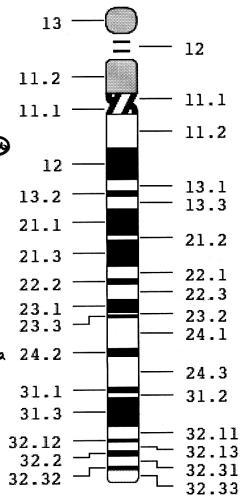


Whole Chr 11 LOH not common.
62% LOH to 11p15.5. 36% SQC.

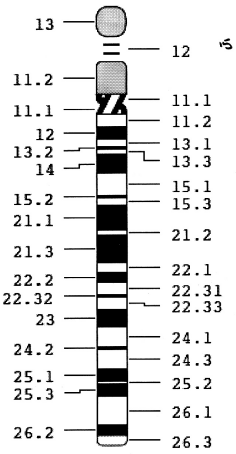
Chromosome 13



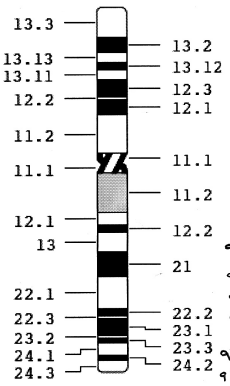
Chromosome 14



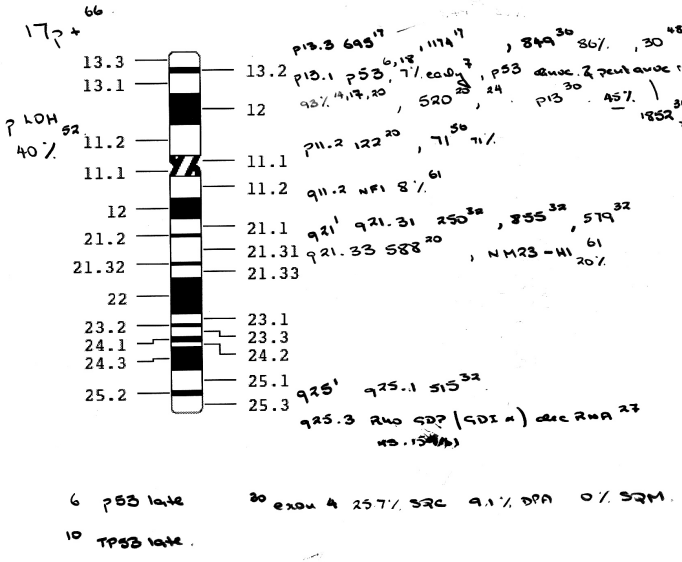
Chromosome 15



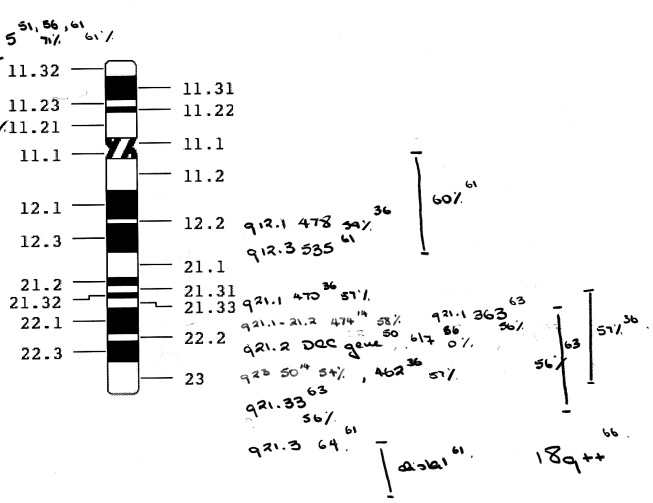
Chromosome 16



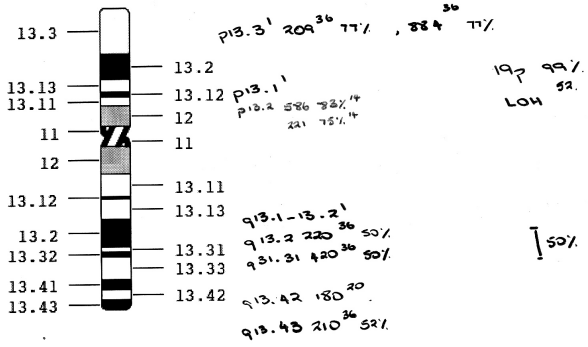
Chromosome 17



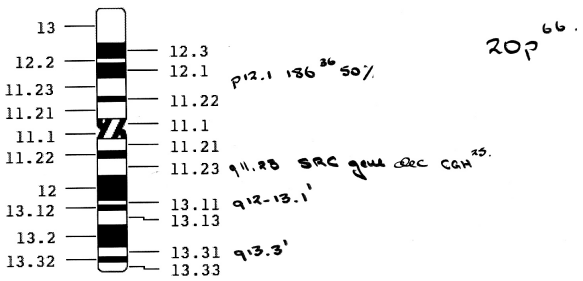
Chromosome 18



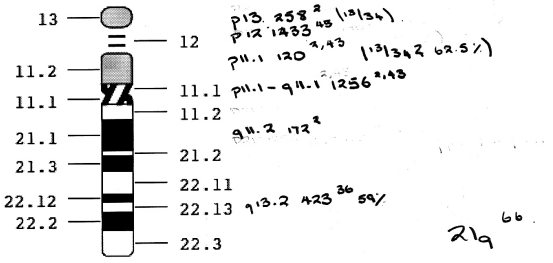
Chromosome 19



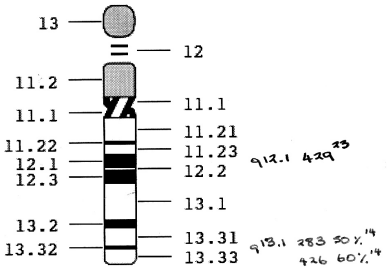
Chromosome 20



Chromosome 21



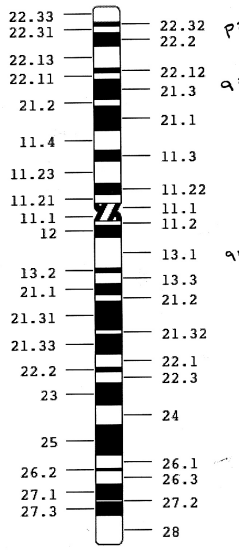
Chromosome 22



LOH 63% JQC

88% NSCLC cell lines.

X Chromosome



XP 66

Chromosome markers from the candidate chromosomal regions were selected using the UniSTS, HGMP and Ensemble databases. The criteria for selection were:

a. A polymorphic microsatellite di-nucleotide repeat marker.
b. Location in the chromosomal region of interest.
c. Size of PCR product < 200 base pairs.
d. Heterozygosity frequency in the general population of > 75% quoted on the Human Genome Database.

The sequences of the primers for these markers were copied from the Human Genome Database (UniSTS and HGMP GDB) and manufactured by Sigma-Genosys™. In each patient, DNA was extracted from blood leucocytes as a source of "normal" patient DNA. The markers selected were assessed for heterozygosity within the patients "normal" DNA. If the marker was homozygous or the 2 alleles were too close together on the gel to facilitate accurate reading then another marker from the same chromosomal location was analysed until a marker of sufficient quality was found.

Loss of heterozygosity analysis protocol

20μl PCR reactions were set up using the protocol in box 4.1. The forward primer was labelled with ^{32}PdATP using T4 polynucleotide kinase. The PCR reactions were run on a thermal cycler with a heated lid for 35 cycles using Amplitaq gold™ DNA polymerase with a 10 minute 95°C activation/denature step, and a 10 minute 72°C final extension step.

Box 4.1 The PCR protocol for 20μl reactions

	Stock concentration	Concentrations in reaction	Totals (μl)
water			**9.85**
Labelled primer			**2.00**
forward primer (20uM)	20μM	0.35μM	**0.35**
reverse primer (20uM)	20μM	0.4μM	**0.40**
Reaction buffer (10x)	10x	1x	**2.00**
MgCl2 (25 mM)	25mM	1.5mM	**1.20**
dNTP (2mM)	2mM	0.2mM	**2.00**
template DNA			**2.00**
Taq	5U/μl	1 unit	**0.20**

Polyacrylamide gel electrophoresis

6% denaturing polyacrylamide gels were used to separate the PCR products. A formamide / EDTA stop buffer with boiling of the combined PCR product / buffer solution was used to maintain the DNA in the denatured state. The gels were dried on a standard gel drier with a vacuum pump at 80°C for 1 hour. The gels were then exposed to autoradiograph film for varying time-periods judged to provide the optimum definition of the bands on the gel. Several exposures were made of each gel. The gels were then read by visual inspection.

Patient P6

Introduction

This 61 yrs old male was referred after a conventional white light bronchoscopy for recurrent pneumonia had shown an abnormality in the lingula. A HGL was found in the biopsies. There was a history of smoking 60 cigarettes per day for 47 years, but he had stopped smoking one year prior to referral.

At bronchoscopy, two months after the initial bronchoscopy at the referring institution, there was a single small abnormality visible under white light and autofluorescence in the lingula. This was maintained under observation using white light and autofluorescence bronchoscopy (figure 4.5). The bronchoscopic appearance of the lingula did not change, with abnormal mucosa seen under white light and autofluorescence. Biopsies from the lingula at month 2, month 6 and month 14 showed histologically normal epithelium only. At month14, there was abnormal mucosa under white light and autofluorescence in the apical segment of the left lower lobe. Biopsies showed a HGL. Two months later, squamous cell carcinoma was identified in the apical segment of the left lower lobe both on bronchoscopic biopsy and PET scan. This was successfully treated with a left lower lobectomy.

Results of molecular studies

The results of the LOH analysis are shown as raw data in table 4.1 and figure 4.5 and as a schematic diagram in figure 4.6. Specimens from the lingula and the apical segment of the LLL were available for analysis. Fifteen markers from ten loci were analysed in all the specimens. The PCR failed in all the specimens for the marker on 8p22.

Lingula

The extent of the LOH in the HGL at month 0 was 4/9 loci. There was LOH at only one of the four 3p loci tested (3p21) and 4p16, 5q15 and 9p21. The other loci analysed retained heterozygosity (3p12, 3p14, 3p25, 5q22 and 17p13).

Table 4.1: Raw data LOH studies of patient P6. The numbers refer to the alleles present on the LOH study. A missing number means that there was LOH at that allele. The histology numbers of the specimens, their bronchoscopic location and the month into the study when they were obtained is shown.

	Specimen no	H02 17502	2003 125	2003 126	2003 131	2003 129	2003 162
	Location	Lingula	Lingula	Lingula	Lingula	LLL apical	LLL apical
	Histology	HGL	NAD	NAD	NAD	CIS	CIS/SQC
Locus	**Marker**	0	2	6	14	14	16
3p12	**D3S 3633**	1,2	1,2	1,2	1,2	1,2	1,2
3p14	**D3S 2318**	1,2	1,2	1,2	1,2	1	1
	D3S 1228	1,2	1,2	1,2	1,2	1,2	,2
3p21	**D3S 2409**	,2	1,2	1,2	1,2	1	1
	D3S 1573	1,2	1,2	1,2	1,2	,2	,2
3p25	**D3S 1293**	1,2	1,2	1,2	1,2	1	1
	D3S 1298	1,2	1,2	1,2	1,2	1,2	1,2
4p16	**D4S 2935**	1	1,2	1	1,2	1,2	1,2
	D4S 394	1	1	1	1	1,2	1
5q15	**D5S 592**	1	1,2	1,2	1,2	,2	,2
	D5S 421	1	1,2	F	1,2	1	1
5q22	**D5S 644**	1,2	1,2	,2	1,2	1,2	1,2
8p22							
9p21	**D9S 171**	,2	,2	F	1,2	,2	,2
17p13	**D17S 1574**	1,2	1,2	,2	1,2	1,2	1,2

Figure 4.5: Raw data of LOH analysis of patient P6. The data are shown by the chromosome marker used, and the histology number of the lesion from which the sample analysed was taken.

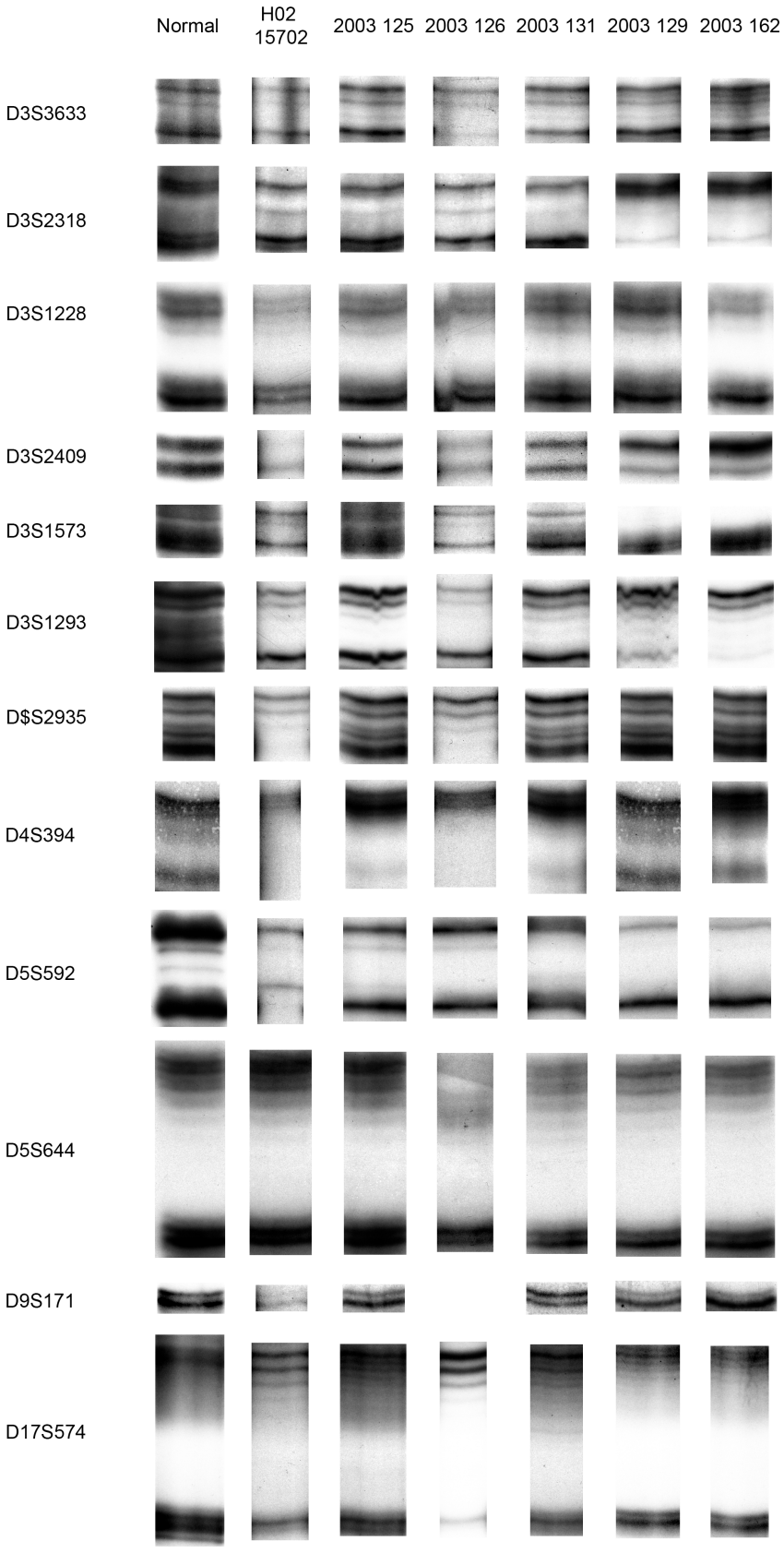
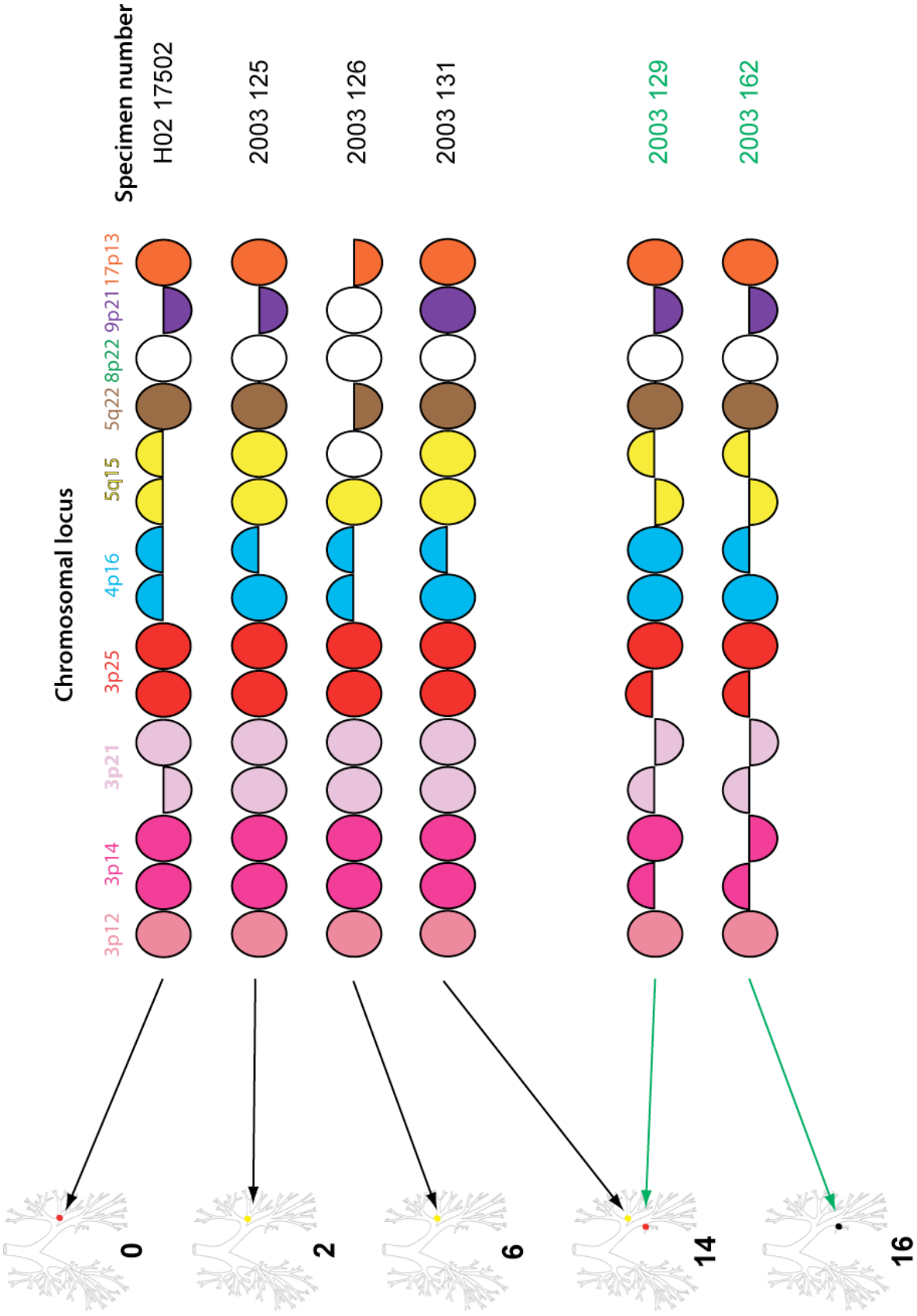


Figure 4.6: Schematic diagram of bronchoscopy findings and LOH findings in patient P6. Each oval represents the results of LOH analysis at a single marker, the colour represents the locus analysed. The top half absent shows LOH at the upper allele, and the lower half absent shows LOH at the lower allele. The arrows show the location in the bronchial tree and in the natural history where the samples were taken.



Biopsies from the lingula at months 2, 6 and 14 showed histologically normal epithelium suggesting regression of the HGL. The extent of the LOH in these specimens was less, at 2-3/9 loci, than the HGL. There was retention of heterozygosity in markers that had shown LOH in the HGL (D3S 2409 at 3p21 and both markers on 5q15) and LOH in markers that retained heterozygosity in the HGL (5q22 and 17p13 in the month 6 specimen). At D4S 394 on 4p16, the same allele was lost in the HGL and in the subsequent biopsies. In the other marker on 4p16, D4S 2935, there was LOH in the HGL but retention of heterozygosity in the subsequent biopsies except the month 6 specimen, in which there was LOH at the same allele as the HGL.

In the lingula samples at months 2, 6 and 14 there was LOH at the same allele at D4S 394 on 4p16. The rest of the allelotypes show that they did not derive from each other as the pattern of LOH is different in each sample. The month 2 specimen had LOH at 4p16 and 9p21. The month 6 specimen had LOH at both markers on 4p16, and additionally at 5q22 and 17p13. The sample from month 14 had LOH at one marker on 4p16 only. This suggests that although the cells in the samples may have had a single progenitor, they had developed different mutation patterns and were therefore from different clones.

Left lower lobe apical segment

The extent of the LOH in the HGL at month 14 was 5/9 loci and was comparable with that of the HGL in the lingula. There was LOH at three of the four 3p loci analysed (4/7 of the markers) and LOH at 5q15 and 9p21. At D3S 2409 on 3p21 the LOH was at allele 1 in the lingula, but allele 2 in the LLL apical segment. Allele 2 was lost at D5S 592 on 5q15 in the lingula segment HGL, but allele 1 was lost in the LLL apical segment. The same alleles were lost in both the lingula HGL and the LLL apical segment HGL at D5S 421 on 5q15 and D9S 171 on 9p21. There was more extensive 3p LOH in the LLL apical segment HGL, with LOH at 3p14, 3p21 (both markers) and D3S 1293 on 3p25. In the specimen from the lingula HGL at month 0 both markers showed LOH at 4p16 whereas in the LLL apical segment at month 14 heterozygosity was retained at that locus.

The LLL apical segment HGL progressed histologically to squamous cell carcinoma after 2 months, and was visible as an area of increased activity on PET scan. The pattern of allele loss in the carcinoma was identical to the HGL in all the markers that had shown LOH in the HGL (D3S 2318 on 3p14, 3p21 both markers, D3S 1293 on 3p25, 5q15 both markers and 9p21). There was retention of heterozygosity in both the HGL and the carcinoma at 3p12, D3S 1298 on 3p25, D4S 2935 on 4p16, D5S 644 on 5q22 and 17p13. Within the carcinoma there was expansion of the 3p LOH to 5/7 markers (although still 3/4 loci) with additional LOH at D3S 1228 on 3p14. There was new LOH on 4p16 (D4S 394) in the carcinoma, which had not been observed in the preceding HGL. This suggests that the carcinoma developed directly from the high-grade lesion at the same bronchoscopic location but in the transition from HGL to invasive carcinoma the extent of the 3p LOH had increased, and there was new LOH at 4p16.

Discussion

The allelotypes suggest that the LLL apical segment HGL and the lingula HGL derived from different progenitor cells and were not clonally related. The lingula lesion showed relatively little 3p LOH, only one marker out of seven showing allele loss, with LOH at 4p16, 5q15 and 9p21, a pattern associated with regression of the HGL. The LLL apical segment HGL had more extensive (5/7 markers) 3p LOH, with 9p21 and 5q15 LOH. This pattern, with the acquisition of the 4p16 LOH, appeared to drive the HGL to invasive carcinoma. The most striking difference between the lingula HGL that regressed, and the LLL apical HGL that progressed to invasive carcinoma was the extent of the 3p LOH. It is possible that extensive 3p LOH, with 9p LOH and 4p16 LOH is required to generate a fully invasive phenotype and avoid regression of the lesion.

Patient P8

Introduction

This 61 year old male was referred to his local unit with haemoptysis. He had smoked 40 cigarettes per day for 48 years and there was a history of asbestos exposure. Conventional white light bronchoscopy showed abnormal mucosa in the RLL apical segment, biopsies from which showed a HGL. One year later in our unit, under white light and autofluorescence, there was the previously noted RLL apical segment lesion and an abnormality in the RUL. Both were histologically HGL. A CT and PET scan did not demonstrate a significant abnormality. Two subsequent bronchoscopies performed at months 17 and 24 showed bronchoscopic and histological regression of both abnormalities to normal epithelium (figure 4.7). The haemoptysis had resolved soon after the first bronchoscopy, and the patient remained asymptomatic throughout.

Results of molecular studies

The results of the LOH analysis are shown as raw data in table 4.2 and figure 4.8 and as a schematic diagram in figure 4.7. Specimens from the RLL apical segment and the RUL were available for analysis. Fifteen markers from ten loci were analysed in all the specimens.

Right lower lobe apical segment

From this location four specimens were analysed, two showing HGL at month 0 and month 12 and two from subsequent histologically and bronchoscopically normal epithelium at month 17 and month 24.

The extent of the LOH was 5/10 loci in the HGL at month 0 and month 12. In the markers showing LOH, the same allele was lost in both samples suggesting that they may be from the same lesion (D3S 2318 on 3p14, D3S 1573 on 3p21, 9p21 and D17S 1574 on 17p13). There was retention of heterozygosity at 3p25, 4p16, 5q22 and 8p22 in both specimens. There were differences between the two specimens. The LOH at 5q15 at month 0 was not present at month 12, and the extent of the LOH at 17p13 increased from month 0 to

month12, with one marker showing LOH at month 0 but both markers involved at month 12. The 3p12 marker was not interpretable as the PCR failed in the month 0 sample.

In the two subsequent specimens, both of which showed normal histology, the extent of LOH was 2/7 loci at month 17 and 3/10 loci at month 24, which is less than in the preceding HGL. The month 17 specimen results are difficult to interpret due to the high number of failed PCRs. At month 24 there was LOH at 3p25, 9p21 and 17p13. The allele lost at one of the markers on 17p13 was different to that of the preceding HGL, and 3p25 showed LOH which was not present in either of the HGL samples. This suggests that the bronchoscopically and histologically normal epithelium at month 24 was not clonally related to the previous HGL.

Right upper lobe

A sample taken at month 17 with histologically normal epithelium that was taken from the location of a previous HGL was analysed. Unfortunately, the original HGL specimen had no remaining lesion cells, and so DNA was not available for study. Of the informative loci, two failed to provide results suitable for analysis (3p12 and 17p13) due to failed PCR.

The extent of the LOH was 3/8 loci (4/12 markers), which is similar to the extent of LOH in the RLL apical segment specimens that showed normal histology after histological regression of the HGL. There was LOH at 3p21, 3p25 and 4p16, in contrast to the histologically normal RLL apical segment specimens (3p25, 9p21 and 17p13), but the same allele was lost at 3p25. Heterozygosity was retained at 9p21, 5q15, 5q22 and 8p22. The allele lost at 3p21 was the same as the HGL in the RLL apical segment but there was LOH at 3p25, 4p16 and the other marker on 3p21(D3S 2409), which was not seen in the HGL.

Table 4.2 : Raw data LOH studies of patient P8. The numbers refer to the alleles present on the LOH study. A missing number means that there was LOH at that allele. The histology numbers of the specimens, their bronchoscopic location and the month into the study when they were obtained is shown.

	Specimen no	WALES	2003 3	2003 49	2004 12	2003 50
	Location	RLL apical	RLL apical	RLL apical	RLL apical	RUL control
	Histology	HGL	HGL	NAD	NAD	NAD
Locus	**Marker**	0	12	17	24	17
3p12	**D3S 1254**	F	,2	F	1,2	F
3p14	**D3S 2318**	1	1	F	1,2	1,2
	D3S 1228	1,2	1,2	1	1,2	1,2
3p21	**D3S 2409**		1,2		1,2	,2
	D3S 1573	1	1	1	1,2	1,2
3p25	**D3S 1293**	1,2	1,2	1,2	,2	,2
4p16	**D4S 2935**	1,2	1,2	1,2	1,2	1
	D4S 394	1,2	1,2	1,2	1,2	1,2
5q15	**D5S 592**	1	1,2	1,2	1,2	1,2
5q22	**D5S 644**	1,2	1,2	1,2	1,2	1,2
8p22	**D8S 136**	1,2	1,2	1,2	1,2	1,2
	D8S 133	1,2	1,2	F	1,2	1,2
9p21	**D9S 162**	,2	,2	F	,2	1,2
17p13	**D17S 1176**	1,2	,2		1	
	D17S 1574	1	1	F	1	F

Figure 4.7: Schematic diagram of bronchoscopic and LOH results from patient P8. Each oval represents the results of LOH analysis at a single marker, the colour represents the locus analysed. The top half absent shows LOH at the upper allele, and the lower half absent shows LOH at the lower allele. The arrows show the location in the bronchial tree and in the natural history where the samples were taken.

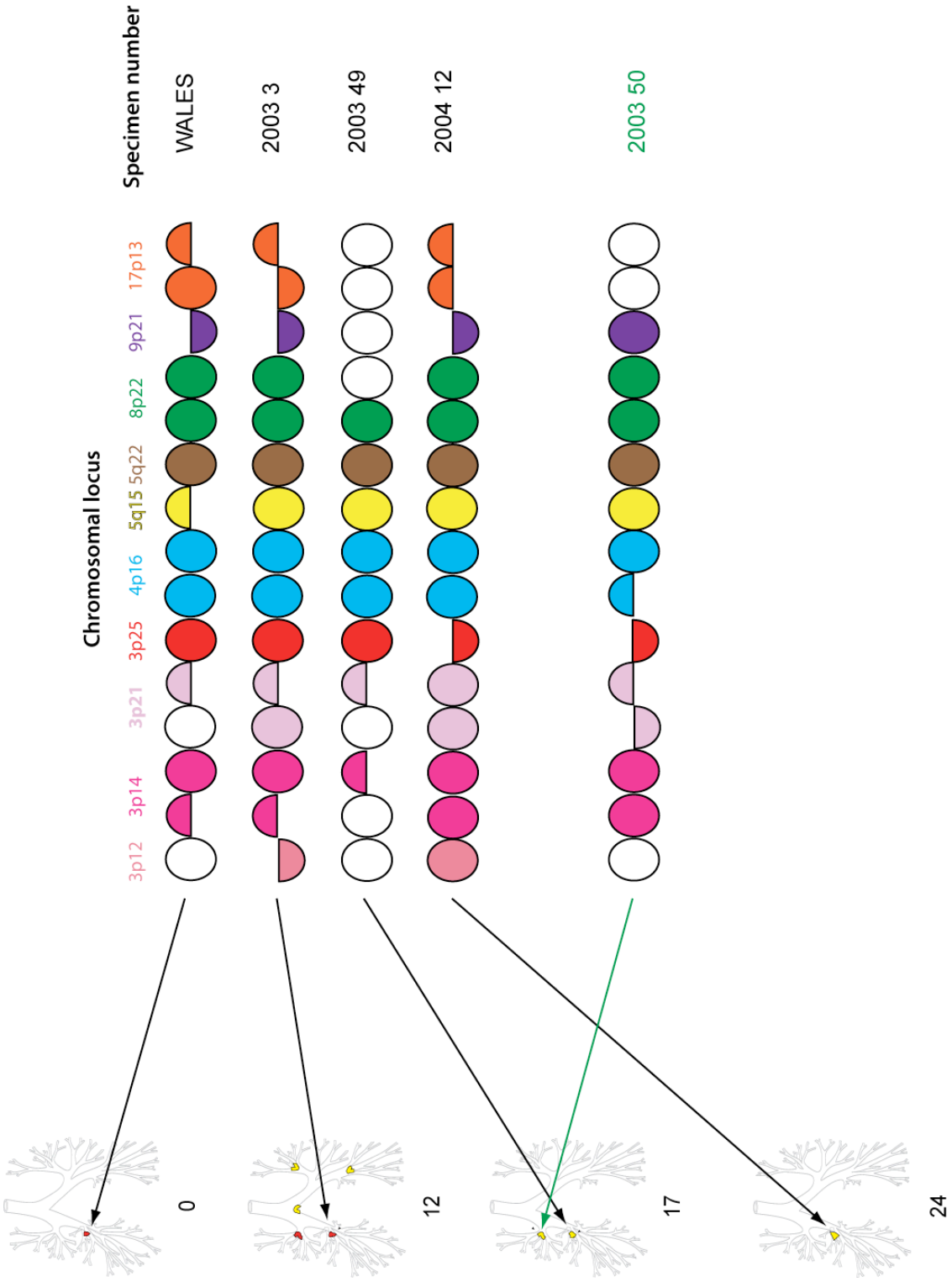
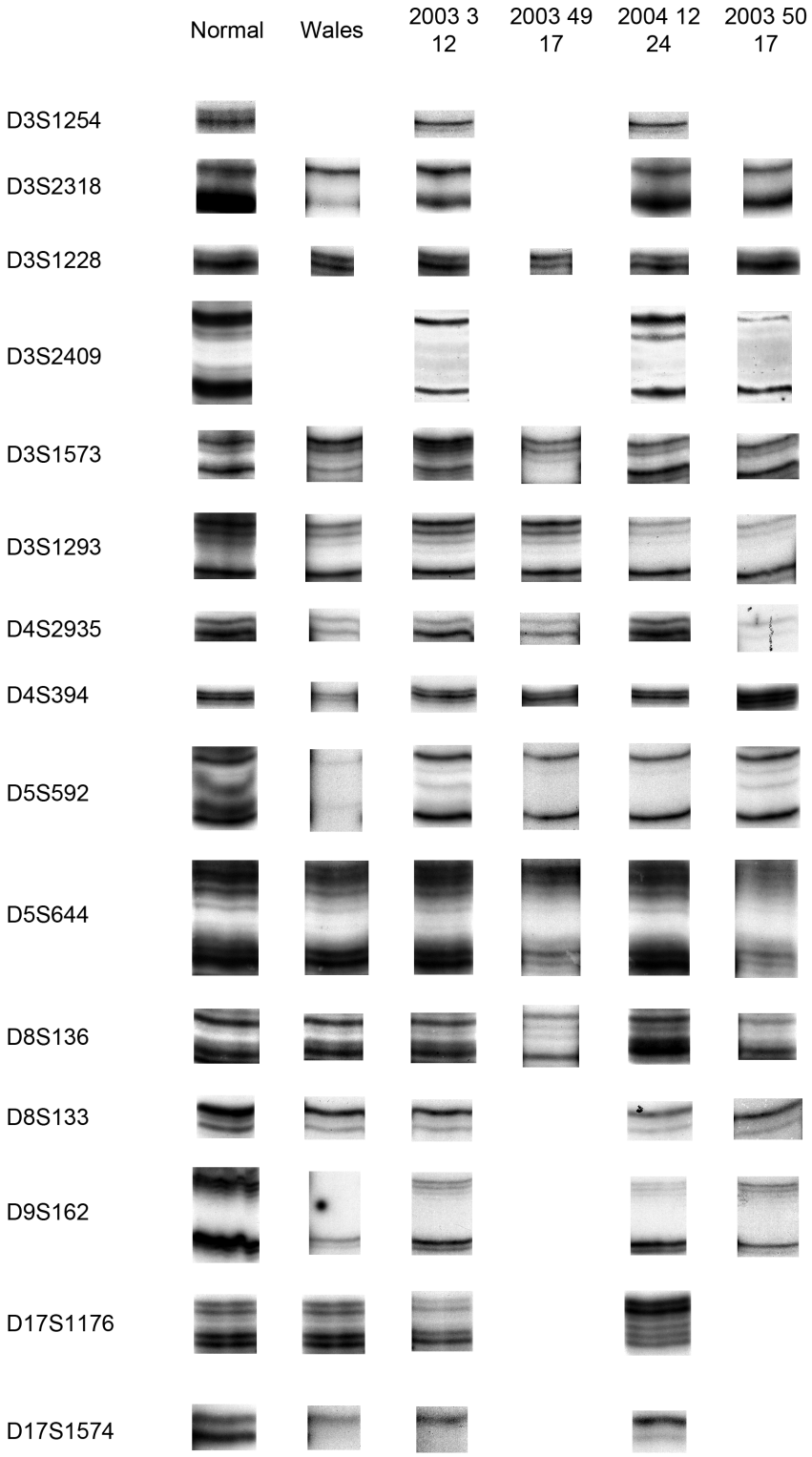


Figure 4.8: Raw data of LOH analysis of patient P8. The data are shown by the chromosome marker used, and the histology number of the lesion from which the sample analysed was taken.



Discussion

There are differences between the allelotypes of the specimens from the RLL apical HGL. The specimen at month 12 had LOH at an additional marker on 17p13 but the LOH at 5q15 in the month 0 specimen was not present. This suggests that the lesion at month 12 did not evolve directly from the month 0 lesion. It is more probable that although both specimens had the same cell of origin, there was clonal divergence i.e. the original lesion cells evolved into separate clones with different molecular changes (figure 4.7). This implies that there may be heterogeneity within the HGL, with clones of cells within the lesion having a single common progenitor but subsequently evolving different molecular changes. The concept of lesion heterogeneity is discussed in more detail in section 4 of the main discussion.

The sample from the RUL at month 17 was from the location of a previous HGL. It is unfortunate that the previous HGL was not available for analysis, as there was no lesion left within the remaining specimen block, suggesting that this HGL may have been completely resected during biopsy. This is borne out in the LOH analysis, in which the allelotype did not resemble that of the HGL in the RLL apical segment or other previously analysed HGLs in other patients. This suggests that the bronchoscopically and histologically normal epithelium at month 24 may not have been clonally related to the previous HGL.

Patient P12

Introduction

This 61yr old male was referred to his local unit with haemoptysis. Three years earlier he had undergone a LUL lobectomy for squamous cell carcinoma after which there had been intermittent haemoptysis. Conventional bronchoscopy one year after the lobectomy had shown no abnormality. A further conventional bronchoscopy at the referring institution two years later had identified abnormal mucosa at the LUL stump, biopsies from which showed a HGL. The patient had stopped smoking at the time of the lobectomy, having smoked 30 cigarettes per day for 35 years.

Bronchoscopy was performed 5 months later at our unit using white light and autofluorescence. There was abnormal mucosa around the LUL stump visible with both imaging modalities, biopsies from which showed a HGL. In subsequent follow-up bronchoscopies between months 5 and 33 the area of bronchoscopically abnormal mucosa increased in size to extend from the proximal LMB to the orifice of the LLL (figure 4.9). The autofluorescence appearance of the mucosa worsened showing increased nodularity. Biopsies of the bronchoscopically abnormal mucosa showed HGLs. Histologically, the appearance of the HGL at the LUL stump changed over time to show epithelial out-pouching into the subepithelial stroma (figure 4.10). This was not seen in the proximal LMB. The LLL orifice was bronchoscopically and histologically normal. The bronchoscopic and histologic appearance of the LUL stump led to a completion left pneumonectomy at month 54. Within the resection specimen a nodule of invasive carcinoma was found at the LUL stump.

Results of molecular studies

The results of the LOH analysis are shown as raw data in table 4.3 and figure 4.10 and as a schematic diagram in figure 4.9. Samples from the LUL (the original carcinoma), the LUL stump, the proximal LMB and the orifice of the LLL were available for analysis with a biopsy of histologically and bronchoscopically normal epithelium from the RUL. Eighteen markers from ten loci were analysed in all the specimens.

Table 4.3 Raw data LOH studies of patient P12. The numbers refer to the alleles present on the LOH study. A missing number means that there was LOH at that allele. The histology numbers of the specimens, their bronchoscopic location and the month into the study when they were obtained is shown.

	Specimen no	**597.9266**	**500.6251**	**R02 019**	**R02 028**	**R02 021**	**2003 44**	**2003 61**	**2003 42**	**2003 40**	**2003 59**
	Location	**LUL**	**LUL**	**LUL stump**	**LUL stump**	**LUL stump**	**LUL stump**	**LLL**	**LLL**	**L main**	**RUL**
	Histology	**SQC**	**SQC**	**HGL**	**HGL**	**HGL**	**HGL**	**NAD**	**HGL**	**HGL**	**NAD**
Locus	**Marker**			**5**	**9**	**14**	**33**	**0**	**33**	**33**	**0**
3p12	D3S 1284	1,2	1	1	1	1	1,2	1,2	1	1	1,2
3p14	D3S 1228	1,2	1,2	X	X	X	X	X	X	X	X
	D3S 2318	1,2	1,2	1	1	1	1	1	1,2	1,2	1,2
	D3S 1233	X	X	F	1	1	1	1	1,2	1	,2
	D3S 1234	X	X	,2	,2	,2	,2	,2	1,2	,2	1,2
3p21	D3S 2409	,2	,2	X	X	X	X	X	X	X	X
	D3S 1573	1	1	1,2	1	1	1	1	1	1	1,2
3p25	D3S 1293	1,2	1,2	1	1	1	1,2	1	1,2	1	1,2
4p16	D4S 2935	X	X	1,2	1,2	1,2	1	1,2	1,2	1	1,2
5q15	D5S 394	X	X	F	,2	1,2	,2	,2	,2	,2	1,2
	D5S 592	1	1	1	1	1	1	1	1	1	1,2
5q22	D5S 644	1	1	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
	APC			1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
8p22	D8S 133	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
	D8S 136	1,2	1,2	,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
9p21	D9S 162	1	1,2	,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
	D19S 1749	2	,2	,2	,2	,2	,2	1,2	1,2	1,2	1,2
17p13	D17S 1574	x	x	1	1,2	1,2	1,2	1,2	1,2	1,2	1,2

Figure 4.9: Schematic diagram of bronchoscopic and LOH results from patient P12. Each oval represents the results of LOH analysis at a single marker, the colour represents the locus analysed. The top half absent shows LOH at the upper allele, and the lower half absent shows LOH at the lower allele. The arrows show the location in the bronchial tree and in the natural history where the samples were taken.

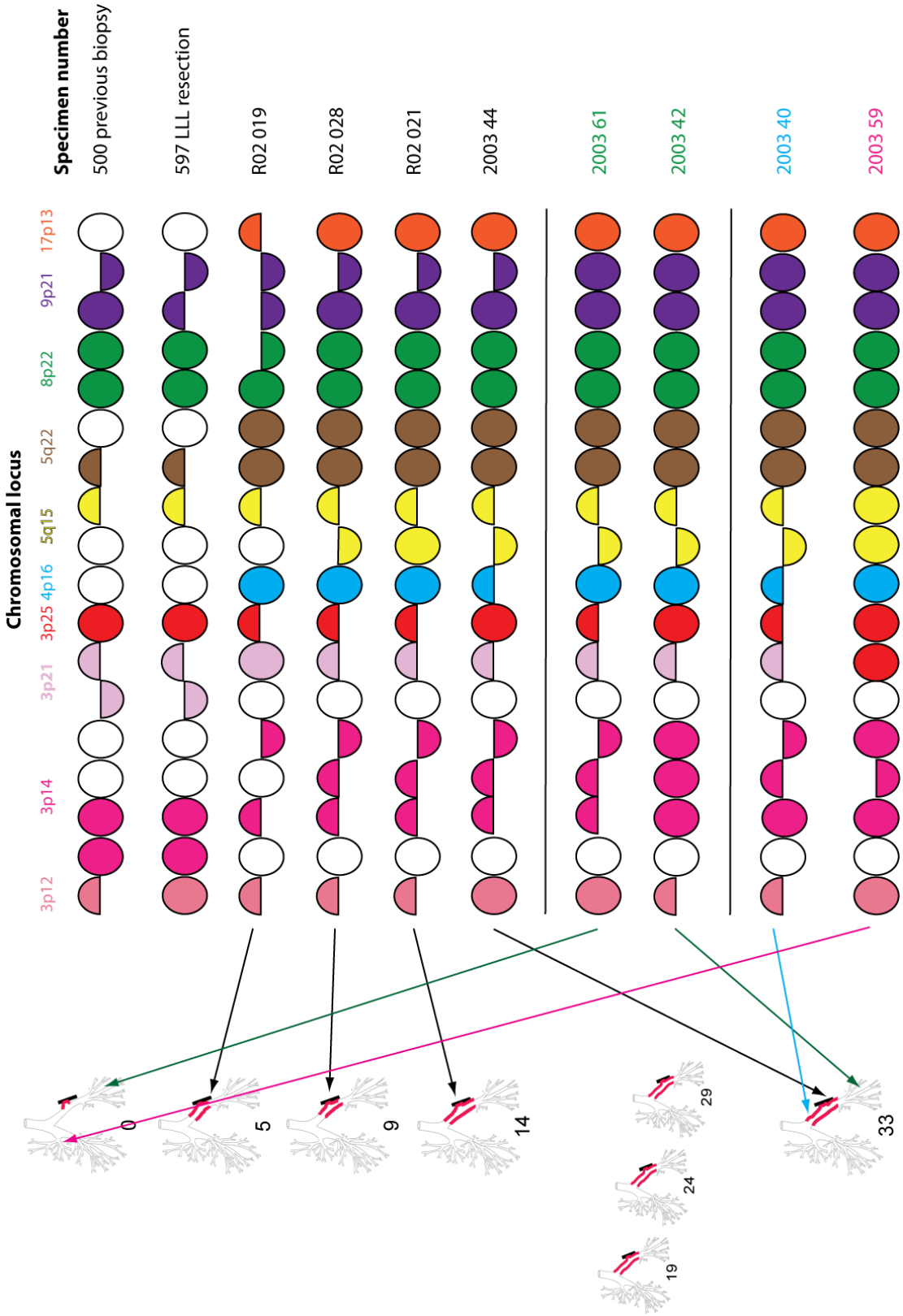


Figure 4.10A: Raw data of LOH analysis of patient P12. The data are shown by the chromosome marker used, and the histology number of the lesion from which the sample analysed was taken.

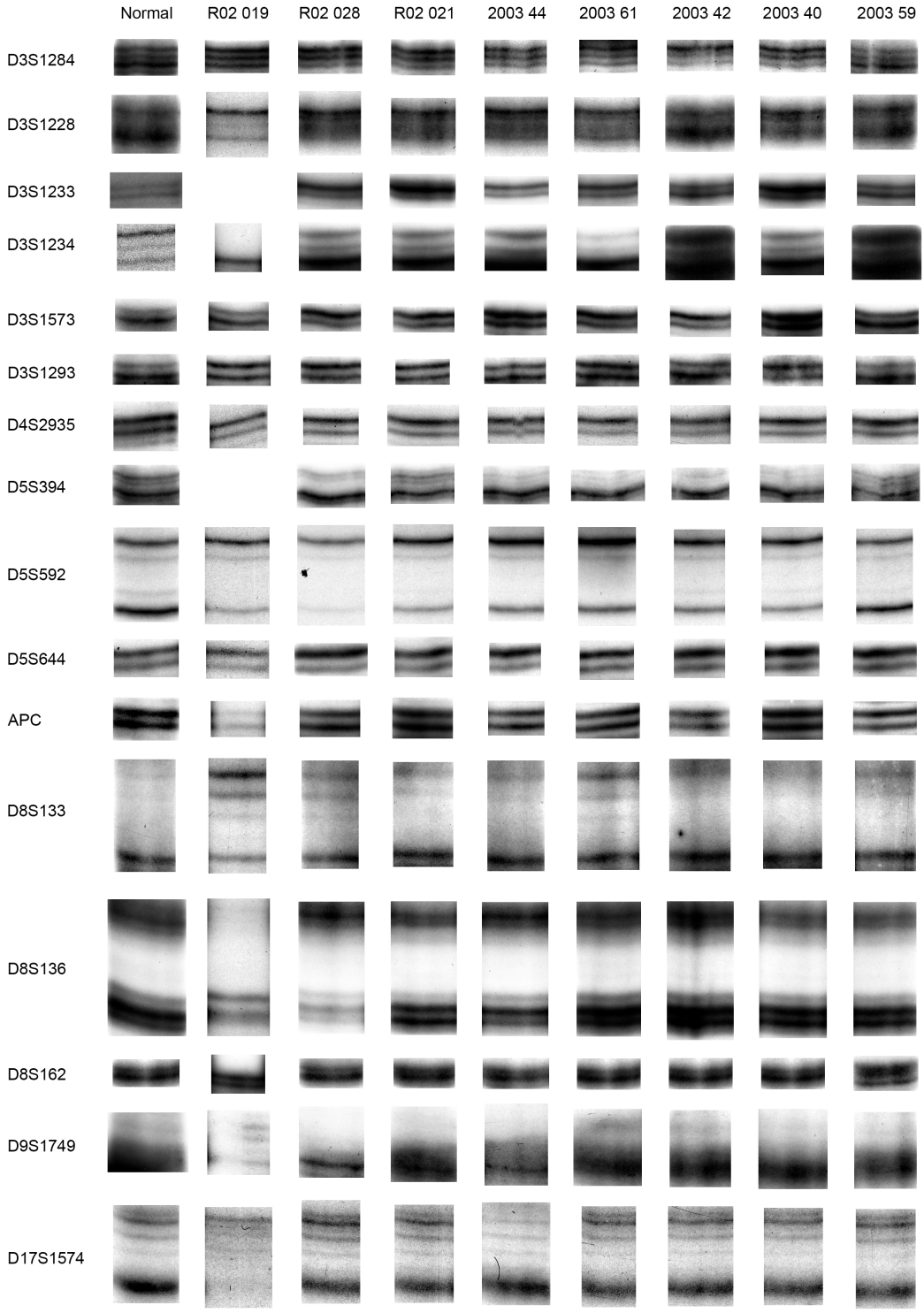
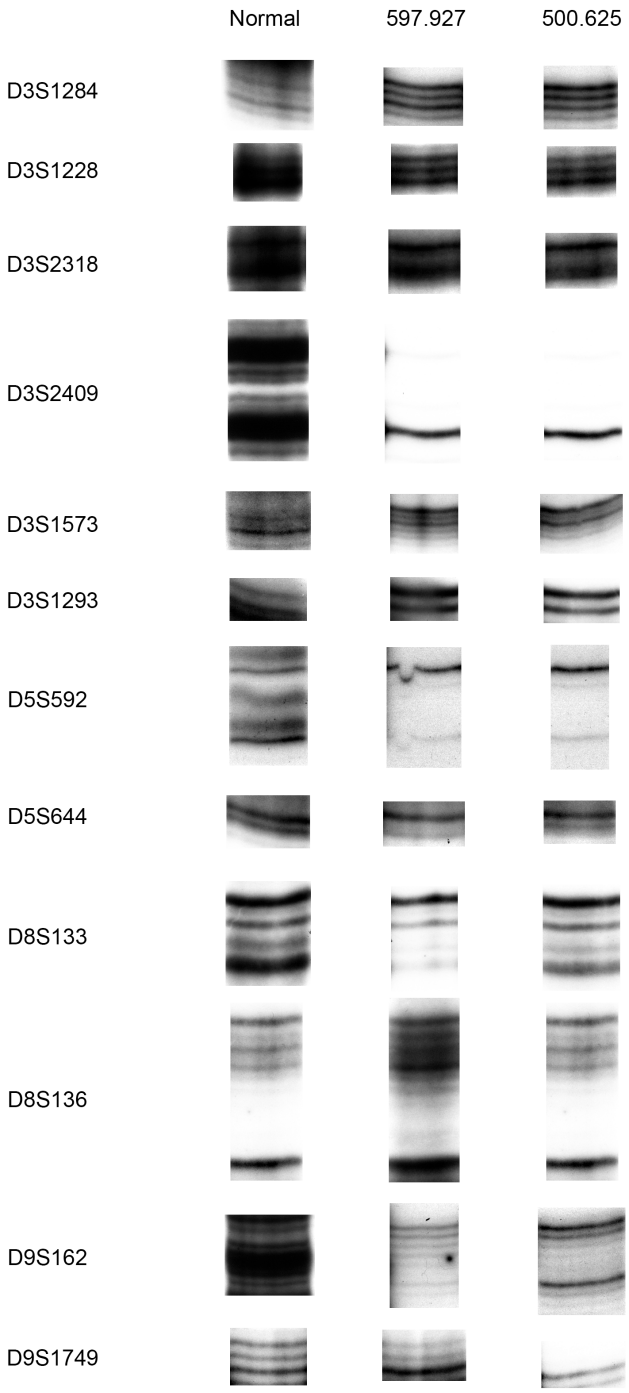


Figure 4.10B: Raw data of LOH analysis of patient P12. The data are shown by the chromosome marker used, and the histology number of the lesion from which the sample analysed was taken.



Left upper lobe carcinoma (the "original carcinoma")

The bronchoscopic biopsy and the resection specimen from the LUL carcinoma were analysed. The results are limited by the failure of the PCR for the markers on 4p16 and 17p13 despite multiple attempts. The extent of the LOH was 5/8 loci (6/12 markers) in the biopsy specimen, and 4/5 loci (6/12 markers) in the resection specimen. There was LOH at the same alleles in both the biopsy and the resection specimen at 3p21 (both markers), 5q15 (D5S 592), 5q22 (D5S 644) and 9p21 (D9S 162). In the resection specimen there was more extensive 9p21 LOH (involving additionally D9S 1749) but there was retention of heterozygosity at 3p12 which had shown LOH in the biopsy specimen.

Left upper lobe stump

Month 5 biopsy: LOH was found at 7/10 loci (9/14 markers). There was extensive 3p LOH (3p12, 3p14 and 3p25) with LOH at 5q15, 8p22 (D8S 136), 9p21 (both markers) and 17p13 and retention of heterozygosity at 3p21, 4p16, 5q22 and 8p22 (D8S 133).

Month 9 biopsy: LOH was found at 6/10 loci (9/16 markers). There was retention of heterozygosity at loci that had shown LOH at month 5, specifically 8p22 (D8S 136), 9p21 (D9S 162) and 17p13. There was new LOH at 3p21 and LOH at 5q15 (D5S 394) for which the PCR had failed in the month 5 specimen.

Month 14 biopsy: The LOH pattern seen in the month 9 specimen was maintained in the month 14 sample, but with retention of heterozygosity at 5q15 (D5S 394) where previously there had been LOH.

Month 33 biopsy: The allelotype of the month 33 biopsy was similar to the preceding samples but showed LOH at fewer loci (5/10 loci, 8/16 markers). There was retention of heterozygosity at 3p12 and 3p25, loci that had shown LOH in the preceding specimens. There was LOH at 5q15 (D5S 394) similar to the month 9 sample, and LOH at 4p16 that had not been present in any of the earlier specimens.

The HGL remained histologically the same diagnosis throughout, although there was epithelial out-pouching into the stroma. An invasive carcinoma was found within the LUL stump HGL during histological examination of the completion pneumonectomy at month 54.

The allelotypes of the LUL squamous cell carcinoma and the subsequent LUL stump HGL were compared. This was limited by the failure of PCR of markers at 3p14, 4p16 and 17p13 within the specimens from the carcinoma. Nevertheless, the extent of the LOH was greater in the LUL stump HGL (7/10 loci) than the carcinoma (5/8 loci). This was largely due to 3p; in the carcinoma two of the four loci showed LOH compared to all four loci in the stump HGL. There was LOH at the same alleles in both the carcinoma and the LUL stump HGL at 3p12, 3p21 (D3S 1573), 5q15 (D5S 592) and 9p21 (D9S 1749). In the month 5 sample from the LUL stump HGL, a different allele was lost at one of the 9p markers (D9S 162) compared to the carcinoma. In the subsequent specimens from the LUL stump HGL there was retention of heterozygosity at this marker. There was LOH at 5q22 (D5S 644) in the carcinoma that was not found in the specimens from the HGL.

Left main bronchus

The specimen from this location was taken when the LUL stump HGL had extended bronchoscopically into the LMB. The extent of the LOH in the LMB was similar to the LUL stump specimens (6/10 loci analysed). Comparing the allelotypes of the LMB and LUL stump samples, the same allele was lost at 3p12, 3p14 (2 out of 3 markers), 3p21, 3p25, 4p16 and 5q15. However, there was retention of heterozygosity in the LMB specimen at one of the 3p14 markers (D3S 2318) and the 9p21 marker (D9S 1749) that had shown LOH in all of the LUL stump samples. The LMB HGL had not progressed to squamous cell carcinoma prior to the completion pneumonectomy.

Left lower lobe

Samples from the bronchoscopically and histologically normal mucosa of the LLL taken 33 months apart were analysed. The extent of the LOH was 4/10 loci in the month 0 specimen and 3/10 loci in the month 33 specimen, less than that of the LMB HGL and the LUL stump HGL (6-7/10 loci). In those markers showing LOH in the LLL the alleles lost were the same as the LMB and LUL stump, 3p12, 3p14, 3p21, 3p25, and 5q15.

There were differences in the allelotypes of the LLL specimens. The month 0 specimen showed extensive 3p LOH at 3p14 (three markers) and LOH at 3p21 and 3p25. In contrast there was LOH at 3p12 and 3p21 but not 3p14 or 3p25 in the month 33 specimen. This suggests that although the cells of both specimens had the same progenitor, there were different clones of cells in the samples at month 0 and month 33.

Right upper lobe

This location was bronchoscopically remote from the left bronchial tree abnormalities, was bronchoscopically normal under both white light and autofluorescence, and contained histologically normal epithelium. There was LOH at one locus (3p14) of ten in the analysis and only one marker of the sixteen analysed. At this marker, the LOH was in a different allele to the allele lost in the LUL stump, LLL, and LMB specimens. This suggests that the cells forming this area of epithelium were not clonally related to the cells of the other bronchoscopic locations sampled.

Discussion

The LUL stump was abnormal with a HGL that extended bronchoscopically to involve the LMB but not the LLL over 33 months. The specimens from the LUL stump showed similar but not identical patterns of LOH with differences between the allelotypes of each specimen. The reasons for this genetic heterogeneity of lesions are not clear, but are discussed in more detail in section 4 of the main discussion.

Left upper lobe carcinoma

The allelotypes of the two specimens from the original LUL carcinoma showed similar LOH patterns at 3p21, 5q15 and 5q22 but differences at 3p12 and 9p21. The discrepancy could be due to sampling error, as one specimen was a bronchial biopsy and the other a resection specimen. The specimens were accurately microdissected, and only tumour cells were used in the analysis which should have minimised the risk of contamination of the specimen with cells of a clone different to the lesion cells. Only 1 month had elapsed between the 2 specimens, making it unlikely that the allelotype of the entire tumour had changed. The

observed LOH changes suggest that there may have been genetic heterogeneity within the carcinoma, with differences in the extent or pattern of the molecular abnormalities in different parts of the lesion. This phenomenon has been observed in other lung tumours[126] but the mechanism by which this may occur is not known but is discussed in part 4 of the main discussion.

Left main bronchus

The bronchoscopic abnormality at the LUL stump appeared to extend to involve the left main bronchus (LMB), from which specimens showed a HGL. Intuitively, it would seem that the LMB lesion was an extension of the LUL stump lesion. This is not supported by the molecular studies. The LMB HGL had LOH at 3p, 4p16 and 5q15 but no LOH at 9p or 3p14 (D3S 2318). During follow-up carcinoma did not develop in this bronchoscopic location. The LUL stump specimens showed LOH at 3p, 9p21 and 5q15 and the acquisition of 4p16 LOH at month 33 was associated with progression to invasive carcinoma. These results suggest that although the LMB HGL and the LUL stump HGL may have had the same progenitor, the LMB HGL did not evolve directly from the LUL stump HGL, but were two different lesions formed from different clones of cells with different outcomes.

The lesions may either have grown towards each other along the bronchial epithelium, or the LMB lesion may have developed from the original progenitor stem cell of the LUL stump HGL, with migration of the cells along the bronchial epithelium. The evolution of this clone would generate an allelotype similar to that of the LUL stump HGL, but with significant differences as observed in the molecular studies. Further study would be required to determine the origin of the LMB lesion.

Patient P4

Introduction

This 62 year old male presented to his local unit with haemoptysis. There was a past history of Chronic Obstructive Pulmonary Disease, Type II Diabetes Mellitus, a high alcohol intake and 53 pack-years of smoking. Conventional white light bronchoscopy showed abnormal mucosa in the LUL, biopsies from which contained a LGL. At follow-up bronchoscopy performed 4 months later there was bleeding at the origin of the LUL biopsies from which showed a HGL.

At our institution 6 months later (month 10) the mucosa of the LUL carina was abnormal under both white light and autofluorescence, although the abnormality appeared more extensive under autofluorescence than white light (figure 4.11). A HGL was found in biopsies from this location. A CT scan of the Thorax at this time showed no lesions. There was no change in the bronchoscopic and histologic appearance of the LUL at months 16 and 22. At month 22 there was abnormal mucosa under autofluorescence but not white light in the LLL. Biopsies showed a LGL. The patient stopped smoking at month 25.

Bronchoscopies performed at months 27, 32, 43 and 50 showed progressive resolution of the LUL bronchoscopic abnormality. The histology showed a LGL in the LUL at month 27, and then normal epithelium from months 32-50. There was regression of the previous LGL to normal epithelium in the LLL at months 27-50. At month 27, a CT scan showed a 1 cm nodule in the LUL, separate from the bronchoscopically abnormal mucosa. There was no change in the size or morphology of the nodule 6 months later. At months 50 & 57, biopsies from the orifice of the LUL, which had shown no abnormality under white light or autofluorescence, demonstrated histologically normal epithelium.

Results of molecular studies

The results of the LOH analysis are shown as raw data in table 4.4 and figure 4.12 and as a schematic diagram in figure 4.11. Specimens from the LUL taken at months 10, 16, 22, 32, 43 and 50 were available for analysis. From the LLL there were biopsies from months 22 and

43, and from the LUL orifice at months 50 and 57. The specimens were analysed using 12 markers from 10 chromosomal loci.

Left upper lobe

The extent of the LOH was 9/10 loci (10/12 markers) in the LUL HGL at month10. The results in the month 16 specimen were compromised by failure of the PCR at 3p21 (D3S 1234) and the month 22 specimen by failed PCRs at 3p14 (D3S 1233), 3p21 (D3S 1234) and 4p16. There was LOH at 3p, 4p16, 5q15, 5q22, 9p21 and 17p13. The same alleles were lost at all the markers that had shown LOH in the RUL HGL specimens (3p12, 3p14, 3p21, 3p25, 5q15, 5q22, 9p21 and 17p13).

There was LOH in all the 3 RUL HGL specimens at 3p12, 3p21, 3p25, 4p16, 5q15 and 5q22. At 8p22, the initial month 10 specimen was heterozygous, but LOH was detected at month 16 and subsequently retention of heterozygosity was found at month 22. At 9p21, the month 10 specimen showed genetic loss, at month 16 there was retention of heterozygosity but at month 22 LOH was observed. The marker at 17p13 showed genetic loss at month 10 and month 16 but retention of heterozygosity at month 22. At 3p14 (D3S 1228) LOH developed between month 10 and 16, and was persistent at month 22.

Bronchoscopically and histologically the LUL HGL appeared to regress to normal epithelium, which was sampled at 3 time-points, months 32, 43 and 50. The extent of the LOH in all three specimens was less than that of the preceding HGL. In the month 32 specimen there was LOH at 6/10 loci (6/12 markers), 2/10 loci (2/12 markers) in the month 43 specimen, and 1/10 loci (1/12 markers) at month 50. The allelotypes of these samples differed from the prior high-grade lesion samples. Different alleles were lost at 3p21 (D3S 1573), 3p25 and 4p16 at month 32. At month 43, there was LOH at 3p14 (D3S 1233) and 17p13, although the alleles lost at these markers were similar to the previous HGL. The allelotype differed from the sample of month 32, as there was retention of heterozygosity at 3p12, 3p21 (D3S 1573), 3p25, 4p16 and 8p22. The month 50 sample showed allele loss at only one locus, 4p16. At

this locus the same allele was lost as the high-grade lesion samples but this was a different allele to that lost in the histologically normal sample at month 32.

Left lower lobe

The left lower lobe was first sampled at month 22 because of a bronchoscopic abnormality (histologically LGL) and at month 43 (bronchoscopically and histologically normal). The extent of the LOH was 5/10 loci (6/10 markers, as two had failed PCR) in the month 22 LGL specimen, and 6/10 loci (7/12 markers) in the month 43 sample. In the month 22 sample there was LOH at 3p12, 3p21 (both markers), 3p25, 9p21 and 17p13. In the month 43 sample, there was LOH at 3p14 (both markers), 3p21 (D3S 1573), 4p16, 5q22, 9p21 and 17p13. In the month 43 sample compared to the month 22 sample, there was retention of heterozygosity where there had previously been LOH at 3p12, 3p21 (D3S 1234) and 3p25. Markers that had shown retention of heterozygosity at month 22 showed LOH at month 43, i.e. 3p14 (D3S 1228) and 4p16. These data suggest that the normal epithelium found at month 34 was not clonally related to the LGL at month 13.

Lingula

There was no bronchoscopic or histological abnormality in the LLL orifice which was sampled as a “control” specimen. There was no LOH detected in the specimen taken at month 50. At month 57 LOH was found at 3p12, 3p14 (D3S 1233) and 17p13. No bronchoscopic or histological abnormality has been observed in the lingula during the year since that sample was obtained.

Table 4.4: Raw data LOH studies of patient P4. The numbers refer to the alleles present on the LOH study. A missing number means that there was LOH at that allele. The histology numbers of the specimens, their bronchoscopic location and the month into the study when they were obtained is shown.

	Specimen no	R02 077	R02 067	R01 260	R01 1625	2003 57	2003 55	R01 190	2003 53	2003 58	2003 46
	Location	LUL	LUL	LUL	LUL	LUL	LUL	LLL	LLL	Lingula	Lingula
	Histology	HGL	HGL	HGL	NAD	NAD	NAD	LGL	NAD	NAD	NAD
Locus	**Marker**	10	16	22	32	43	50	22	43	50	57
3p12	D3S 1284	1	1	1	1	1,2	1,2	1	1,2	1,2	1
3p14	D3S 1228	1,2	1	1	1,2	1,2	1,2	1,2	1	1,2	1,2
	D3S 1233	1	1	F	1,2	1	1,2	F	1	1,2	,2
3p21	D3S 1234	,2	F	F	1,2	1,2	1,2	,2	1,2	1,2	1,2
	D3S 1573	,2	,2	,2	1	1,2	1,2	1	1	1,2	1,2
3p25	D3S1298	,2	,2	,2	1	1,2	1,2	,2	1,2	1,2	1,2
4p16	D4S2946	,2	,2	F	1	1,2	,2	1,2	,2	1,2	1,2
5q22	DS5592	1	F	1	1,2	1,2	1,2	F	1	1,2	1,2
5q15	D5S644	,2	,2	,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
8p22	D8S136	1,2	1	1,2	1	1,2	1,2	1,2	1,2	1,2	1,2
	D8S133	1,2	1,2	1,2	1,2	1,2	1,2	1	1,2	1,2	1,2
9p21	D9S1749	,2	1,2	,2	,2	,2	,2	1,2	1,2	,2	1,2
17p13	D17S1574	1	1	1,2	1	1	1,2	1	1	1,2	1

Figure 4.11: Schematic diagram of bronchoscopic and LOH results from patient P4. Each oval represents the results of LOH analysis at a single marker, the colour represents the locus analysed. The top half absent shows LOH at the upper allele, and the lower half absent shows LOH at the lower allele. The arrows show the location in the bronchial tree and in the natural history where the samples were taken.

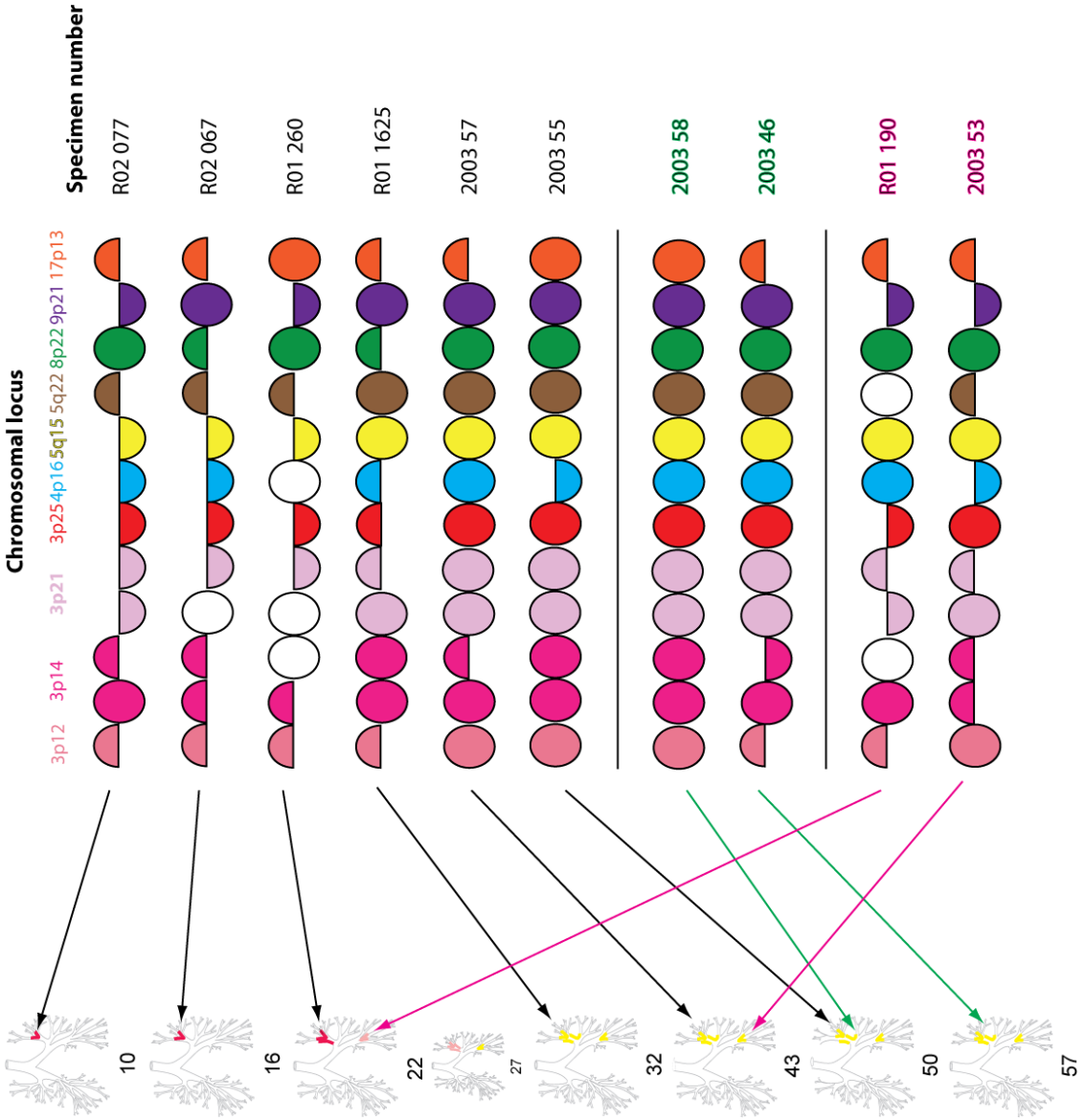
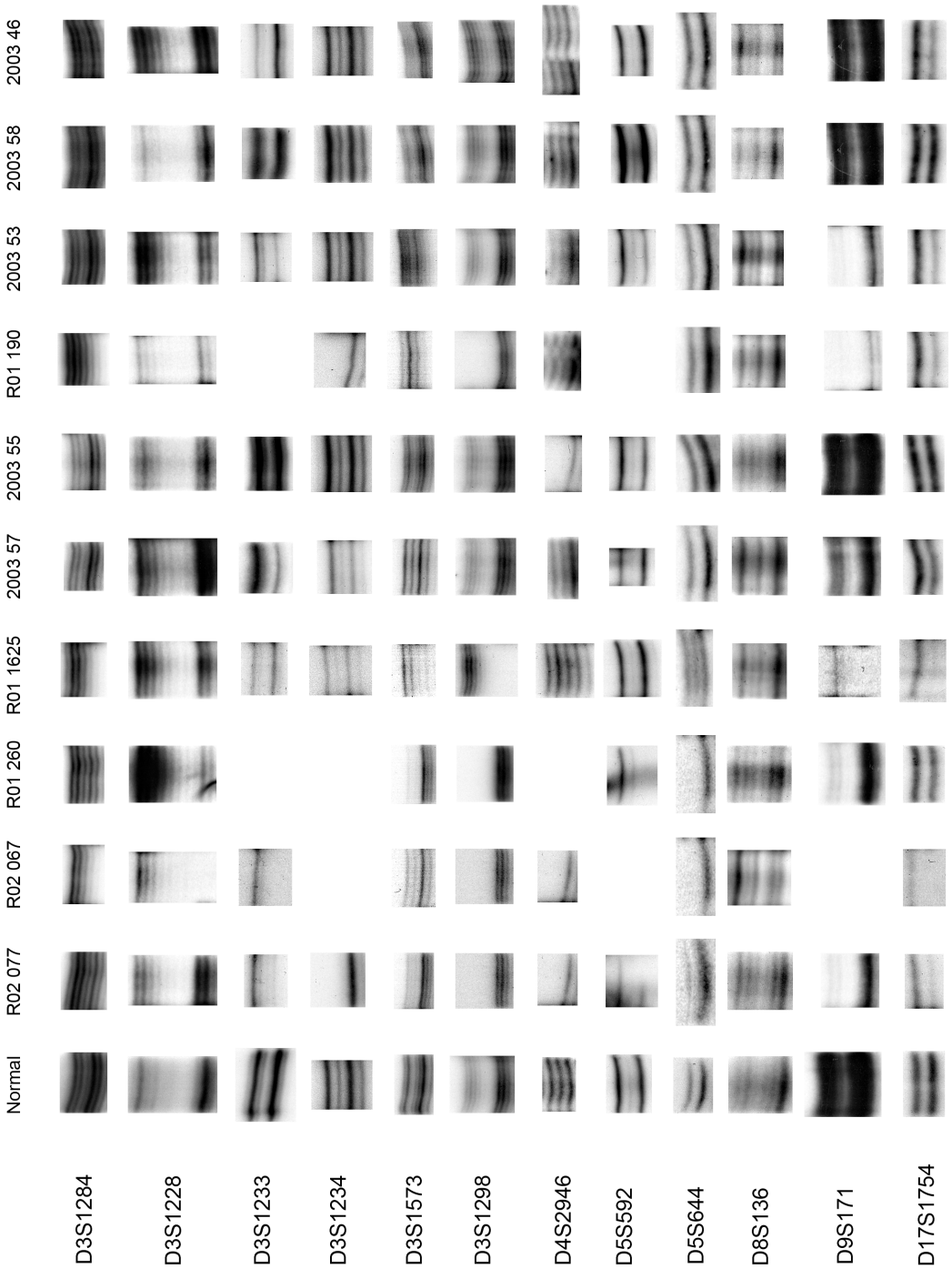


Figure 4.12: Raw data of LOH analysis of patient P4. The data are shown by the chromosome marker used, and the histology number of the lesion from which the sample analysed was taken.



Discussion

Bronchoscopically and histologically the LUL HGL regressed to histologically normal epithelium over 23 months. During this period the patient stopped smoking. The HGL was sampled at 3 time-points, months 10, 16 and 22. The pattern and extent of allele loss in the samples were similar to that found in squamous cell carcinomas in previous studies. Histological and bronchoscopic regression of the high-grade lesion was associated with retention of heterozygosity in 2 of the 3 specimens at 8p22 and in 1 specimen at 9p21 and 17p13. The other chromosomal loci studied, 3p12, 3p14, 3p21, 3p25, 4p16, 5q22 and 5q15 all showed consistent LOH within the specimens from the high-grade lesion. These findings suggest that the retention of genetic material at 8p22, 9p21 and 17p13 may have been associated with the regression of the HGL to normal epithelium despite the presence of extensive genetic damage elsewhere in the genome. This is supported by the retention of heterozygosity at 8p22 and 17p13 in the LLL LGL at month 22 that regressed to histologically normal epithelium. The retention of heterozygosity at 9p21 and 17p13 in the HGL occurred in only 1 of the 3 specimens. It is possible that the retention of genetic material at 8p22, 9p21 or 17p13, or a combination of these loci may influence the regression of pre-invasive lesions. It is similarly possible that these changes are markers of and related to more important changes elsewhere in the genome that drive the behaviour of pre-invasive lesions.

Patient P11

Introduction

This 75 year old patient presented with haemoptysis. Two years earlier he had undergone a LLL lobectomy for squamous cell carcinoma. There was a past history of chronic obstructive pulmonary disease (FEV_1 1.32L, FVC 2.1L), type II diabetes mellitus, benign prostate hypertrophy and diverticular disease. He smoked 40 cigarettes per day for 40 years until the lobectomy. Conventional bronchoscopy found irregular mucosa over the lobectomy stump, biopsies from which showed a HGL.

Bronchoscopy was performed 4 months later at our unit. There was abnormal mucosa adjacent to the lobectomy stump under white light but with autofluorescence the abnormality was more extensive and additionally there was abnormal mucosa in the LUL. Biopsies of both these areas showed HGLs. CT of the thorax showed irregularity of the posterior wall of the left upper lobe bronchus but a PET scan did not demonstrate any increased uptake.

The HGLs were maintained under bronchoscopic follow-up every 4 months (figure 4.13). There was indurated mucosa at the left lower lobectomy stump that was visible under white light and autofluorescence. The LUL HGL was only visible using autofluorescence; there was no discernible abnormality using white light bronchoscopy. The bronchoscopic appearance did not change in extent or severity during this period and histologically, both lesions remained HGLs.

At month 17, there was microinvasion of the stroma in biopsies from the left lower lobectomy stump. A CT-PET scan revealed a synchronous contralateral 2cm diameter lesion in the RLL. Both lesions were squamous cell carcinomas. The RLL lesion was removed by wedge resection and the LLL stump lesion was treated with radical brachytherapy with curative intent. After treatment, bronchoscopic follow-up of the entire visible tracheobronchial tree continued every 4-6 months. The LLL stump was complicated by a post-brachytherapy ulcer with aspergillus colonisation that was successfully treated with itraconazole. No abnormal lesions were found either in the LUL or the LLL stump during the 17 months after completion

of the brachytherapy, between months 27 to 41. In month 41, biopsies from the LUL showed HGL. A CT scan of the thorax showed no abnormality. At month 45, micro-invasion was found within biopsies from the LUL HGL, and this was treated with photodynamic therapy.

Results of molecular studies

The results of the LOH analysis are shown as raw data in table 4.5 and figure 4.14 and as a schematic diagram in figure 4.13. Samples from the LLL carcinoma, the LLL stump HGL and the subsequent microinvasive carcinoma were available for analysis, along with the LUL HGL at months 0 and 17, and the RLL carcinoma. Fourteen markers from ten loci were analysed in all the specimens.

Left lower lobe

The results were limited by the failure of the PCR at 3p21 in all of the specimens. The extent of the LOH was 7/9 loci (10/13 markers) in the LLL carcinoma, 7/9 loci (10/12 markers) in the LLL stump HGL and 6/9 loci (10/12 markers) in the LLL stump carcinoma.

In the LLL carcinoma there was LOH at 3p12 (D3S 1284), 3p14, 3p25, 5q15, 5q22, 8p22 and 9p21. The HGL at the LLL stump had a very similar pattern of LOH to the LLL carcinoma. The only difference was at 3p12 (D3S 3633), which had retained heterozygosity in the carcinoma but showed LOH in the HGL. At month 17, there was a carcinoma at the LLL stump, at the location of the HGL. The allelotype was virtually identical to the HGL but with one important difference. There had been LOH at 8p22 in the LLL carcinoma and the LLL stump HGL. In the LLL stump carcinoma heterozygosity was retained at this locus.

The LLL stump was sampled at month 27, 7 months after brachytherapy. There was LOH at 6/9 loci (6/12 markers). The same alleles were lost at 3p12 (D3S 1284), 3p14(D3S 2318) and 9p21 (D9S 162) as the LLL carcinoma and the LLL stump carcinoma. Different alleles were lost at 3p25, 5q15 (D5S 592) and 5q22.

Left upper lobe

This was sampled at month 0 and month 17, with HGL found in both specimens. At month 0 the results were compromised by the failure of the PCR at 5q15 (D5S 421) and 9p21 (D9S 171). The extent of the LOH was 4/9 loci (4/11 markers) at month 0 and 8/9 loci (12/13 markers) at month 17.

At month 0 there was LOH at 3p12, 3p14 (D3S 2318), 5q15 (D5S592) and 9p21 (D9S162). The alleles lost were identical to the LLL carcinoma, and the LLL stump carcinoma except for 9p21 (D9S162). At this marker a different allele was lost in the LUL HGL compared to both the LLL carcinoma and the LLL stump carcinoma.

At month 17 there was LOH at 3p12, 3p14 (D3S1228), 3p25, 4p16, 5q15, 5q22, 8p22 and 9p21. Detailed analysis of 4p16 showed that the LOH was only at marker D4S2366. The alleles lost were identical to the month 0 HGL except for 9p21 at D9S162, at which different alleles were lost in the month 0 and month 17 specimens. At 8p22 there was LOH at month 17 but retained heterozygosity in the month 0 specimen.

The month 17 sample shows a virtually identical LOH pattern to the previous LLL carcinoma. The only differences are LOH at 3p12 (D3S3633) and 4p16 (D4S2366) that had retained heterozygosity in the LLL carcinoma although there was LOH at D4S 2925 in the LLL carcinoma. Compared to the LLL stump carcinoma, the month 17 HGL showed a virtually identical LOH profile apart from LOH at 4p16 and 8p22 that was heterozygous in the LLL stump carcinoma.

Right lower lobe

This was removed by wedge resection with clear margins at month 17. There was LOH at 8/9 loci (11/13 markers), at 3p12, 3p14, 3p25, 4p16, 5q15, 5q22, 8p22 and 9p21 (D9S171), and all the markers used to characterise the 4p16 LOH in detail.

Compared to the LLL carcinoma, there was a similar LOH profile with the same alleles lost at 3p12 (D3S 1284), 3p14, 3p25, 5q15 (D5S592), 8p22 and 9p21. There were different alleles lost at D4S2925, 5q15 (D5S 421) and 5q22. There was retention of heterozygosity at 4p16 (D4S 2366), and 8p22 in the LLL carcinoma but LOH in the RLL carcinoma

Compared to the synchronous LLL stump carcinoma, there was a similar LOH profile with the same alleles lost at 3p12, 3p14, 3p25, 5q15 (D5S 592) and 9p21 and retention of heterozygosity at 17p13. However, in the RLL carcinoma there was LOH at 4p16 and 8p22 that had shown retention of heterozygosity in the LLL stump carcinoma. Different alleles were lost at 5q15 (D5S421) and 5q22 in the RLL carcinoma compared to the LLL stump carcinoma.

Table 4.5: Raw data LOH studies of patient P11. The numbers refer to the alleles present on the LOH study. A missing number means that there was LOH at that allele. The histology numbers of the specimens, their bronchoscopic location and the month into the study when they were obtained is shown.

	Specimen no	S SQC	R02 023	U02 18635B	2003 99	U01 9745D	U02 18635A	U03 4949
	Location	LLL	LLL	LLL	LLL	LUL	LUL	RLL
	Histology	SQC	CIS	SQC	SD	CIS	CIS	SQC
Locus	**Marker**	Previous	0	17	27	0	17	20
3p12	D3S 1284	,2	,2	,2	,2	,2	,2	,2
	D3S 3633	1,2	,2	,2	1,2	,2	,2	,2
3p14	D3S 1228	,2	,2	,2	1,2	1,2	,2	,2
	D3S 2318	1	1	1	1	1	1	1
3p21	D3S 2409	H	H	H	H	H	H	H
3p25	D3S 1293	,2	,2	,2	1	1,2	,2	,2
4p16	D4S 2366	1,2	1,2	1,2	1,2	1,2	,2	1
5q15	D5S 421	1	F	1,	1,2	F?	1	,2
	D5S 592	,2	,2	,2	1	,2	,2	1
5q22	D5S 644	,2	,2	,2	1	1,2	,2	1
8p22	D8S 133	1	1	1,2	1,2	1,2	1	1
9p21	D9S 171	,2	,2	,2	F	F	,2	,2
	D9S 162	1	1	1	1	,2	1	1,2
17p13	D17S 1749	1,2	1,2	1,2	1,2	1,2	1,2	1,2

Table 4.5B: Specimens from chromosome 4

	S SQC	U02 18635B	U02 18635A	U03 4949
	Previous	17	17	20
D4S 2366	1,2	1,2	,2	1,
D4S 2935	1,2	1,2	1,2	1,
D4S 3023	H	H	H	H
D4S 3034	1,2	1,2	1,2	,2
D4S 431	1,2	1,2	1,2	,2
D4S 2925	1,2	1,2	1,2	,2

Figure 4.13: Schematic diagram of bronchoscopic and LOH results from patient P11. Each oval represents the results of LOH analysis at a single marker, the colour represents the locus analysed. The top half absent shows LOH at the upper allele, and the lower half absent shows LOH at the lower allele. The arrows show the location in the bronchial tree and in the natural history where the samples were taken.

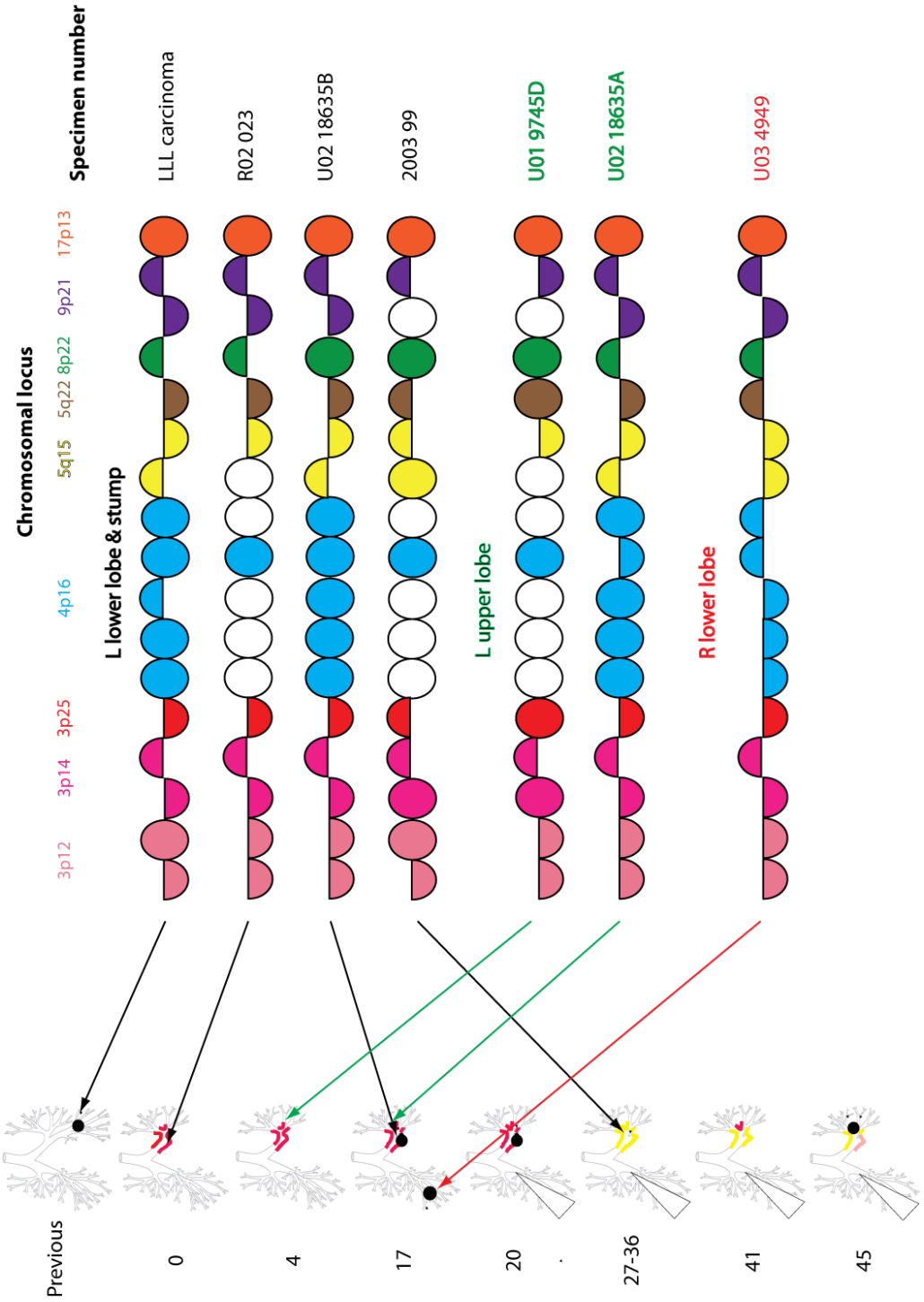
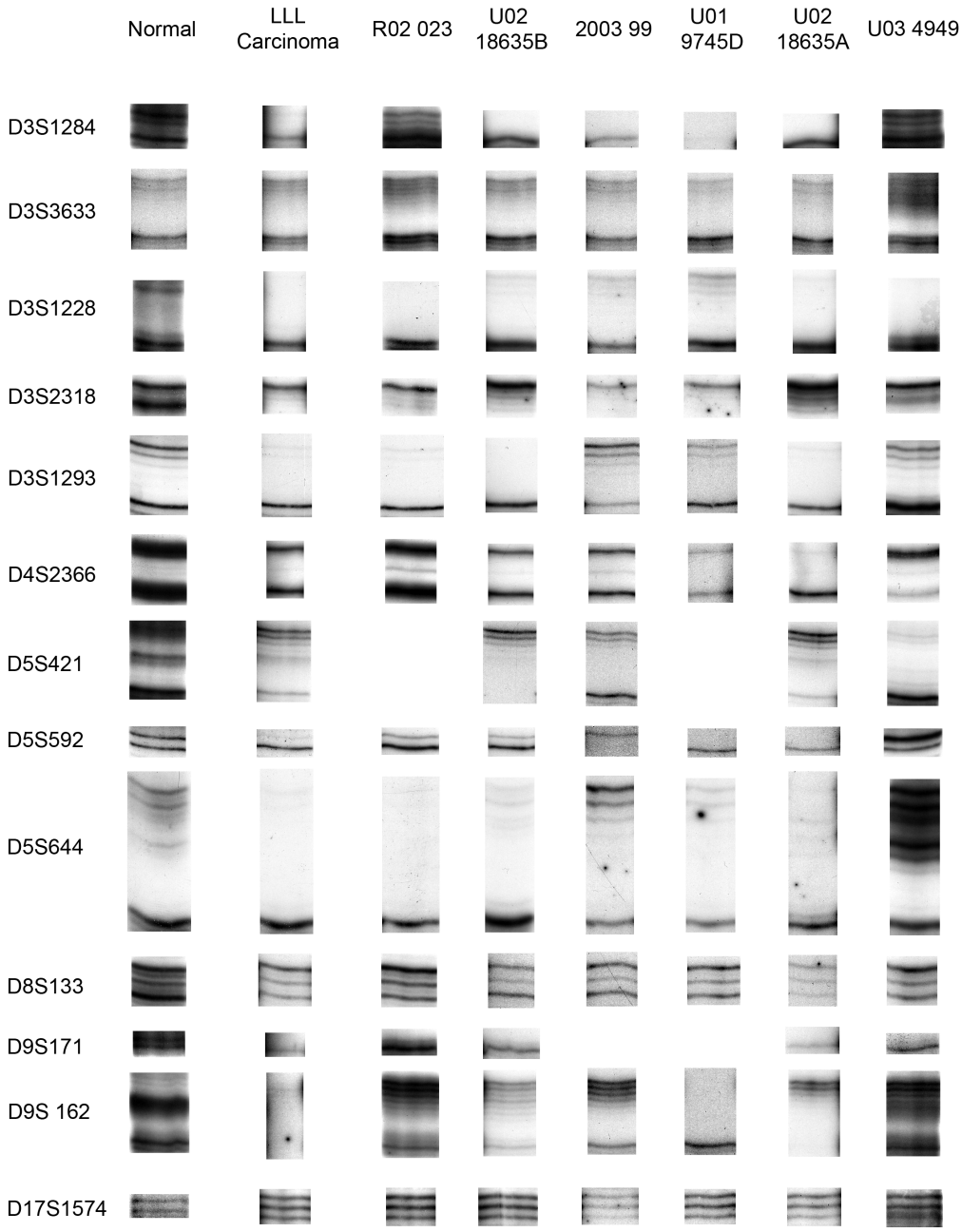


Figure 4.14: Raw data of LOH analysis of patient P11. The data are shown by the chromosome marker used, and the histology number of the lesion from which the sample analysed was taken.



Discussion

The clonal origins of the three carcinomas

The LLL carcinoma and the LLL stump carcinoma

The LLL stump carcinoma arose at the resection margin of LLL carcinoma forty-seven months later and could represent a recurrence of that tumour. The LLL carcinoma and the LLL stump carcinoma had very similar patterns of allele loss with ten out of twelve loci having lost the same allele. This might suggest that the two carcinomas had the same cells of origin.

The LLL carcinoma and the LLL stump carcinoma both showed LOH at D3S1284 whereas the LLL stump carcinoma showed additional loss more proximally on chromosome 3 at D3S 3633. This LOH at D3S 3633 was also seen in the HGL at month 0, that preceded the LLL stump carcinoma. The LLL carcinoma and the HGL at the LLL stump had virtually identical LOH profiles, suggesting that the HGL was related directly to the LLL carcinoma. "Expansion" of the deleted region on chromosome 3 has been observed in association with apparent tumour progression[38,93,104,105]. Thus this pattern of loss is compatible with the LLL stump HGL having developed directly from the LLL carcinoma and the LLL stump carcinoma having developed from a clone within the LLL carcinoma.

A marker at 8p22 that showed LOH in the LLL carcinoma was heterozygous in the LLL stump carcinoma; a configuration that is difficult to explain if the LLL stump carcinoma developed directly from the original carcinoma or the LLL stump HGL. The original LLL carcinoma had D4S 2925 LOH whereas the later occurring carcinoma at the LLL stump had no loss at this locus mirroring the results at 8p22 locus. This suggests that the LLL stump carcinoma did not directly evolve from the original LLL carcinoma or the HGL at the LLL stump but from an earlier progenitor (figure 4.13). One interpretation of these observations is that the origin of these two tumours was essentially clonal but with divergence of related clones with loss around D4S2925 as a late event in the LLL stump carcinoma and loss at D3S 3633 and D8S 133 occurring as a late event in the LLL carcinoma.

The RLL carcinoma

The allelotype of the RLL carcinoma was essentially identical to that of the LLL carcinoma and the LLL stump carcinoma except for the markers on chromosomes 4 and 5. The LOH pattern on 4p in the RLL carcinoma was strikingly different from that of the LLL carcinoma and the LLL stump carcinoma in that all five informative loci show clear LOH. One interpretation is that the RLL carcinoma developed from a clone within the LLL carcinoma or the LLL stump carcinoma in which a region on chromosome 4 was lost resulting in the acquisition of a migratory phenotype and thus the RLL carcinoma represents a metastatic deposit from the LLL carcinoma or the LLL stump carcinoma.

However the clinical observations mitigate against this hypothesis: the RLL carcinoma, is believed to have been another primary tumour. The molecular data support the clinical observations. Although loss of 4p alleles in the RLL carcinoma is consistent with its having developed from the LLL carcinoma or the LLL stump carcinoma i.e. being a metastatic tumour, the actual alleles lost at D4S2925, D5S421 and D5S644 are not from the same homologues in the RLL carcinoma as in the LLL carcinoma or the LLL stump carcinoma implying an independent origin. The allelotypes are incompatible with development of the RLL carcinoma directly from the LLL carcinoma but compatible with the RLL carcinoma having developed from a progenitor lesion that could be the same lesion from which LLL carcinoma and LLL stump carcinoma developed (figure 4.13).

The suggestion of a clonal origin for synchronous and metachronous cancer is not new[127] and the possibility of migrating cancer precursor cells has been discussed particularly with regard to colon cancer[128,129]. More recently this has been elaborated to suggest that the mobile cells are cancer stem cells[130]. In the lung, evidence for mobile epithelial cells has been provided by observation of host engraftment of the bronchial epithelium following lung transplantation[123]. Thus analysis of these tumours suggests that although they are clonally related they may nonetheless represent independent primary tumours. This is an important issue as the degree of allelic congruence shared by these tumours could have been take as evidence that they all originated from the first tumour in the left lower lobe. The LLL stump

carcinoma and the LLL stump carcinoma were treated with curative intent on clinical grounds, and this approach has been supported by the molecular data.

The HGL at the site of the LLL stump carcinoma

The pattern of allelic loss in the HGL that was detected at the site of the LLL stump carcinoma following brachytherapy had more loci scored as heterozygous than either the LLL carcinoma or the LLL stump carcinoma and where there was LOH, the alleles lost were not always the same as those lost in the tumour. Thus this lesion is likely to have arisen from a different progenitor although an early common ancestor cannot be ruled out as the allelic loss that is common to the post-brachytherapy stump HGL and the other LLL lesions was LOH on chromosome 3 and 9, loci where allele loss is predicted to occur as very early events in the development of squamous cell carcinoma[104,105,109,112].

The LUL HGL

The LUL HGL was detected by autofluorescence at month 0 and persisted through to month 17. When the LLL stump HGL and LUL HGL detected synchronously at month 0 are compared by LOH analysis, they share a pattern of common allelic loss having LOH at those loci that are most frequently lost in other lesions in this patient e.g. DS31284, D3S2318 but the LUL HGL has more loci that retained heterozygosity. Seventeen months later, the LUL HGL has additional allele loss with a pattern of loss that is consistent with its having developed from the earlier LUL HGL. There was LOH at month 17 that was not present at month 0, suggestive of progression of the changes in the month 0 lesion. The LOH at 9p21 (D9S162) shows different alleles lost at months 0 and 17. This suggests that the LUL HGL at month 17 may not have developed directly from the month 0 lesion, but from a common early progenitor, or a different clone of cells. The allelotype of the month 17 HGL is similar to both the LLL carcinoma and the LLL stump carcinoma, but with a greater extent of LOH suggesting that it may have evolved from one of these carcinomas. Over further follow-up, a carcinoma was found at the LUL, in the location of the month 17 HGL. In the absence of the molecular data from the carcinoma, it may be concluded that the HGL at the LUL did acquire the malignant phenotype, with the pattern of LOH demonstrated at month 17.

The LLL carcinoma, the RLL carcinoma and the LUL HGL that developed into the LUL carcinoma all shared the pattern 3p and 9p LOH with 4p16 LOH developing pre-invasion. The LLL stump carcinoma did not have LOH in the markers chosen. This may be due to the markers chosen missing the area of LOH in the specimen, or that there was no LOH at 4p16. This might refute the suggestion that the 3p-9p-4p16 LOH pattern is important in the pathogenesis of squamous cell carcinoma or imply that additional molecular changes over and above the 3p-9p-4p16 pattern are required to develop the invasive phenotype.

Discussion of molecular studies

1. Limitations of the techniques used in the molecular studies

LOH analysis is associated with a number of problems in its execution and interpretation[131] but nevertheless is used extensively for the analysis of small biopsies[38,102,103]. There are several issues associated with this technique that may affect interpretation of the results:

a. DNA quality

i. Microdissection

H&E stained sections from the samples selected for analysis were reviewed with a Consultant Histopathologist with a special interest in pulmonary pathology. The section was photographed and the lesion of interest highlighted which facilitated accurate microdissection. Contamination from normal epithelial cells not part of the lesion of interest may have occurred as the boundary of the lesion was not always clearly defined or non-lesion cells which were poorly adherent to the microscope slide may have bound to the Laser Capture Microdissection cap and been removed inadvertently. Although the system is theoretically capable of removing single cells this is not always the case, and cells from around the area of the microdissection may occasionally be removed. In a few lesions, there was intraepithelial invasion by carcinoma-in-situ, producing a heterogeneous lesion which could not be accurately microdissected to remove the lesion cells alone. The protocol was designed to enable the most accurate microdissection of the lesion of interest possible within these limitations. This used conservative microdissections which maintained as clear a boundary as possible between the lesion of interest and non-lesion cells.

A number of the biopsies were disrupted during either the biopsy procedure or in processing which resulted in fragmentation or folding of the epithelium. Intercellular adhesion is known to be reduced in high-grade lesions which increases the risk of loss of the epithelium. In such situations, the cells of the lesion of interest were difficult to identify, and consequently microdissection was less accurate, and may have been contaminated by unwanted cells. It is possible that contamination with non-lesion cells altered the PCR product band pattern and thus compromised the results of the LOH analysis. Any contamination by DNA from non-lesion cells in the PCR reaction would not produce as strong a PCR product band as the

DNA from the lesion of interest due partly to differences in the quantity of DNA template at the start of the PCR reaction. LOH would still be observed, but with a reduction in the intensity of the "lost" PCR product band, rather than a complete absence of the PCR product band. This appearance would be impossible to differentiate from genetically heterogeneous lesions, in which some of the cells have loss of genetic material from a particular chromosomal locus and others do not. Complete loss of an allele at any genetic locus should not occur if there is contamination by non-lesion cells. However, it is still theoretically possible to have complete loss of an allele if the non-lesion epithelium has genetic loss at the same locus. The presence of genetic loss in histologically and bronchoscopically normal epithelium in the present and previous studies means that although unlikely, there is a possibility that this may have occurred. In a number of samples, complete loss of an allele band was seen at some genetic loci with the appearance of reduction in the intensity of one allele at others. To compensate for this in the interpretation of the results, LOH was scored when there was either complete or partial loss of a band when compared to the normal reference DNA.

ii. DNA extraction

There is evidence that using standard histological staining protocols in which there is 5 minute staining with haematoxylin can reduce the yield of DNA from microdissected lesions[132]. This was observed in the pilot stages of the present study (data not presented in this document) and therefore a shorter procedure in which the sections were briefly dipped in haematoxylin was used. This minimised the effects of haematoxylin on the DNA, whilst providing sufficient nuclear staining to enable accurate identification and microdissection of the lesion of interest. Nuclear Fast Red, which in previous studies was not found to affect the quality or quantity of the DNA extracted from biopsy samples[132], was trialled as the stain prior to laser capture microdissection. The stain poorly differentiated the lesion of interest from the surrounding stroma, which made microdissection more difficult and consequently less accurate (data not presented in this document). Pilot studies showed that the method using a short exposure to haematoxylin did not adversely affect the quality of the PCR product or the results of LOH analysis when compared to Nuclear Fast Red (data not

presented in this document). In the absence of an accurate and reliable DNA quantification method this was accepted as evidence that the use of a short exposure to haematoxylin to stain the sections prior to Laser Capture Microdissection would not compromise the results.

b. Polymerase chain reaction and LOH

DNA extracted from the patient's peripheral blood lymphocytes was used as the source of "normal" reference DNA for the analysis. Blood was not available for two patients so stromal cells and lymphocytes were microdissected from the slides from which lesions of interest had previously been microdissected. This particular source of "normal" subject DNA has been used in previous studies of LOH in carcinoma of the bronchus[108]. In the patients in whom this source of "normal" DNA was used, only those polymorphic chromosomal markers that were heterozygous in the stromal DNA were selected, which eliminated the risk that the LOH analysis would be compromised.

The DNA extracted from the lesions of interest was not quantified prior to use in the LOH analyses. In experiments using the Picogreen assay, the buffer used for DNA extraction from the microdissected biopsies produced a variable false positive signal. Alternative DNA extraction buffers were trialled with similar results (data not presented in this document). The results of the quantification were therefore unreliable, and could not be used to define the amount of DNA used in the PCR reactions during LOH analysis. The bronchial biopsies used in the studies were unique, limited in number and small, the median diameter being approximately 1mm. The lesion of interest typically formed less than 10% of the total biopsy. Consequently the total quantity of sample DNA was severely limited and it was decided not to undertake either a purification step or further attempts at DNA quantification, due to the risk of DNA loss during such a procedure. As a result, the quantity of DNA in each PCR experiment was not controlled and this may have affected the results. Different starting quantities of template may alter the dynamics of primer annealing to the sample DNA and alter the products from the PCR. The altered products may affect the interpretation of the bands during LOH analysis. To minimise this, microdissection was performed such that an approximately equal number of cells were obtained from each sample. This could not

eliminate variations in the total quantity of DNA extracted from each biopsy, and hence the amount of DNA used in each PCR experiment, but the extent of that variation was reduced. Furthermore dilution of the DNA further reduced the variation in the quantity of DNA template used in the PCR reactions. The effect of the use of an uncontrolled quantity of DNA template in the PCR reaction on the LOH analysis is not known, and introduced an unquantifiable variable into the experiment.

c. LOH analysis

A visual scoring method was used for the LOH analysis which can be criticised as open to subjective variation in the interpretation of the band patterns. Only those samples in which both the alleles were clearly identifiable on the autoradiograph were evaluated within the study. Samples in which there was difficulty in interpretation or in which the PCR product bands were unclear were excluded from the analysis, or were corroborated using another marker from the same chromosomal location. Prior to use in the LOH analysis, each chromosomal marker was used to investigate the patient's own normal DNA and only those markers showing clear visible PCR product bands and heterozygosity were selected for the analysis of patient samples.

There are systems in which the PCR product bands can be analysed using computer-based imaging. The intensity of the PCR product bands on the autoradiograph are quantified giving an objective measure of the differences between the PCR product bands. Experience from previous studies had shown that computerised analysis provided little additional information, as differences in the bands were reliably identified visually. In the present study, where allele loss was scored as either present or absent, quantification by computerised image analysis, which provides a ratio of the intensities of the 2 PCR product bands, would not alter the result. A fully quantitative method using fluorescent-labelled chromosome primers, and analysis on a DNA sequencing machine was an alternative option[133]. However, the technique selected for the analysis in the present studies had the advantage of having been used successfully for a number of years within the laboratory and a consequent familiarity with the problems, pitfalls and issues regarding interpretation.

Table 4.6: LOH findings in the lesions analysed. The red bars represent LOH within the given lesion at the locus shown. The white bars represent failure of the PCR and therefore no result. The green bars represent retention of heterozygosity at the locus shown. The extent of the LOH is given as a percentage of the total number of loci analysed.

Progression

	Extent LOH	3p12	3p14	3p21	3p25	4p16	5q15	5q22	8p22	9p21	17p13
HGL P6 LLL apical	55										
SQC P6 LLL apical	67										
HGL P12 LUL stump	70										
HGL P11 LUL	90										
SQC P11 LLL	80										
SQC P11 LLL stump	70										
SQC P11 RLL	90										

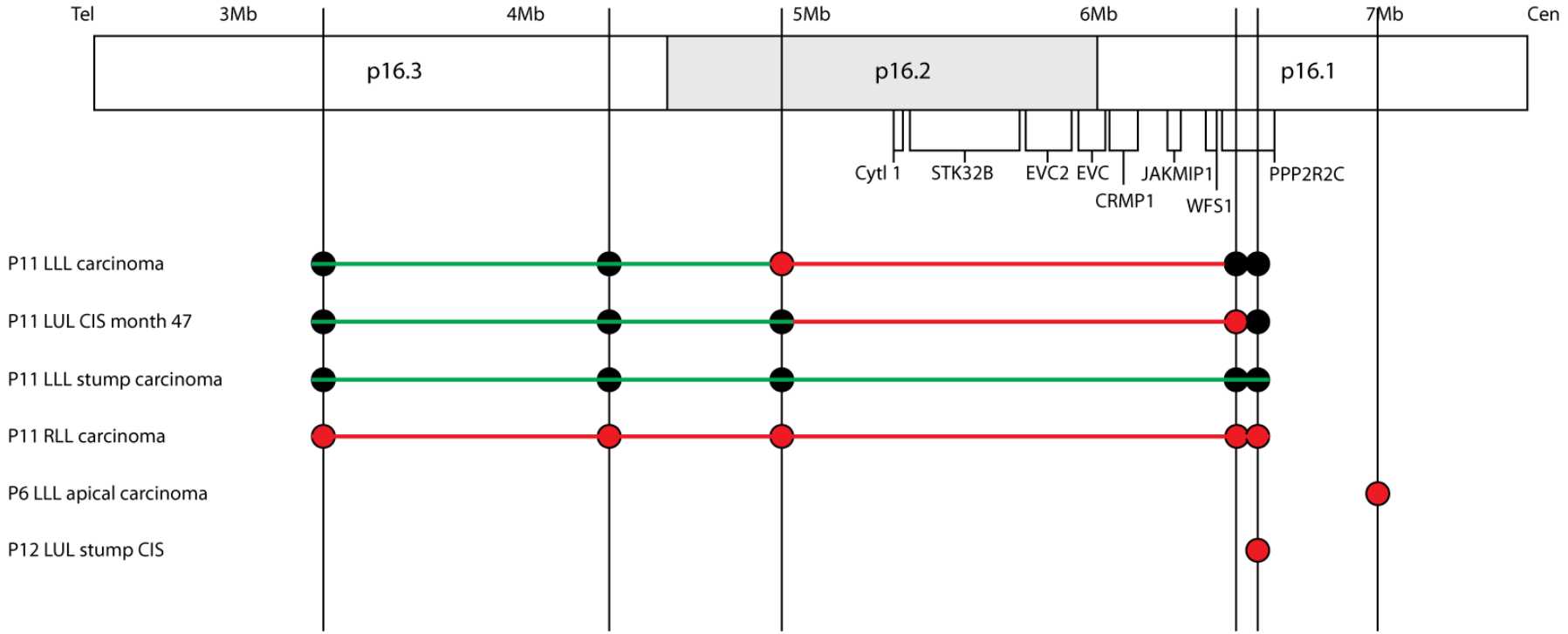
Regression

	Extent LOH	3p12	3p14	3p21	3p25	4p16	5q15	5q22	8p22	9p21	17p13
HGL P8 RLL apical	50										
HGL P6 Lingula	40										
HGL P4 LUL	90										
LGL P4 LLL	50										

No outcome

	Extent LOH	3p12	3p14	3p21	3p25	4p16	5q15	5q22	8p22	9p21	17p13
HGL P12 LMB	60										

Figure 4.15: Schematic diagram of 4p16. On the ideogram of the region, the genes within the minimal region of LOH are shown. The LOH is shown in red, and the green areas represent retention of heterozygosity. The lesions with the LOH shown are shown to the left.



2. Features associated with the progression of pre-invasive lesions to carcinoma

Progression of HGL to invasive carcinoma was observed in 2 lesions in 2 patients (P6 and P12), and 3 separate carcinomas were studied in 1 patient (P11). Table 4.6 shows the LOH findings in the lesions studied.

In the HGLs that progressed to squamous cell carcinoma (P6 HGL LLL apical, P12 LUL stump and P11 LUL HGL) there was extensive LOH at 3p (3 out of 4 loci) and LOH at 9p21 and 5q15. The HGLs at the P12 LUL stump and P11 LUL acquired 4p16 LOH in the sample prior to the identification of invasive squamous cell carcinoma. There was retention of heterozygosity at 4p16 in the HGL at P6 LLL apical. Two months later the squamous cell carcinoma at the same site contained an identical pattern of allele loss to the HGL but with acquisition of 4p16 LOH and extension of the LOH at 3p14. The LLL carcinoma and RLL carcinoma in patient P11 had LOH at 3p (2/3 markers LLL carcinoma and 3/3 markers RLL carcinoma), and LOH at 9p21, 5q15 and 4p16 although there is no sample from a pre-existing HGL for comparison. There are 2 other patients in whom LOH at 4p16 on a background of 3p and 9p LOH has preceded the development of invasive squamous cell carcinoma, reported in a previous publication[117].

Previous studies have shown that LOH at 3p and 9p occur as early events in the development of pre-invasive lesions of the bronchus[104,105,109,112]. This is supported by the finding of 3p and 9p LOH in all but one of the HGL and LGL analysed in the present study. The occurrence of 3p and 9p LOH alone may not drive the progression of an individual lesion to invasive squamous cell carcinoma, as similar changes are seen in lesions that regress to histologically normal epithelium. LOH at 4p16, shown to occur at or around the transition from pre-invasive to fully invasive disease may drive the progression to invasion of an individual pre-invasive lesion.

The HGLs and carcinomas that showed 4p16 LOH were mapped to determine a common minimally deleted region. Figure 4.15 shows that this region lies between marker D4S2925 and D4S2366, a region of 1.44Mb (4944252-6385660). Table 4.7 lists the genes included in

this region and their known functions. Studies in addition to ours have highlighted the association of 4p15-16 loss with the progression from primary to metastatic tumour[134-136].

The minimal region defined by deletion mapping encompasses seven genes (figure 4.15, table 4.7): two are particularly noteworthy with regard to tumour development. PPP2R2C codes for a regulatory subunit of PP2A, a serine/threonine specific phosphatase that interacts directly with small T antigen to cause cell transformation[137]. A number of independent studies indicate PP2A's role in tumour suppressor[138] including the observation that there are inactivating mutations in the gene for another subunit of the PP2A complex, PPP2R1B, in lung and colon cancer[139]. The CRMP1 gene, coding for collapsin response mediator protein, also maps within the minimal deleted region and is an even more attractive candidate for the gene in this region whose loss drives tumour invasion/progression. It is the downstream effector of CTGF (connective tissue growth factor) which has been shown to inhibit invasion and metastases in human lung adenocarcinoma[140].

The exception to this pattern is the P12 LLL stump samples. The HGL at the LLL stump showed LOH at 3p (3/3 loci), 5q15 and 9p21, an allelotype compatible with a spread or recurrence of the LLL carcinoma. The marker used at 4p16, D3S2366, retained heterozygosity in the LLL carcinoma, which on detailed testing showed LOH at another marker, D4S2925. Thus it is possible that there was unrecognized LOH at 4p16 in the HGL at the LLL stump at month 0. The LOH pattern of the LLL stump carcinoma suggested that it had not developed from the HGL at the same location, but from an earlier progenitor. There was no LOH in the 4p16 markers tested in the LLL stump carcinoma. This argues against the 3p-9p-4p16 LOH pattern as an important factor in the development of the LLL stump squamous cell carcinoma, or may suggest that not enough markers were used to adequately exclude LOH at this locus. The weight of evidence from other lesions in the same patient and lesions from four other patients suggest that LOH in this region may be involved in the progression to invasion of a pre-invasive lesion.

Figure 4.16: The progression of genetic damage within pre-invasive lesions. This shows that the progression of pre-invasive lesions from normal epithelium may be related to a. progression of genetic damage within a progenitor clone, b. The progression of genetic damage may not be the same in different clones from the same progenitor and c. May be unrelated to other progenitor clones within the bronchial tree. Each circle represents a genetic locus, loss of the upper half of the circle represents loss of the upper allele and loss of the lower half of the oval represents loss of the lower allele as seen on electrophoresis of PCR products for each locus.

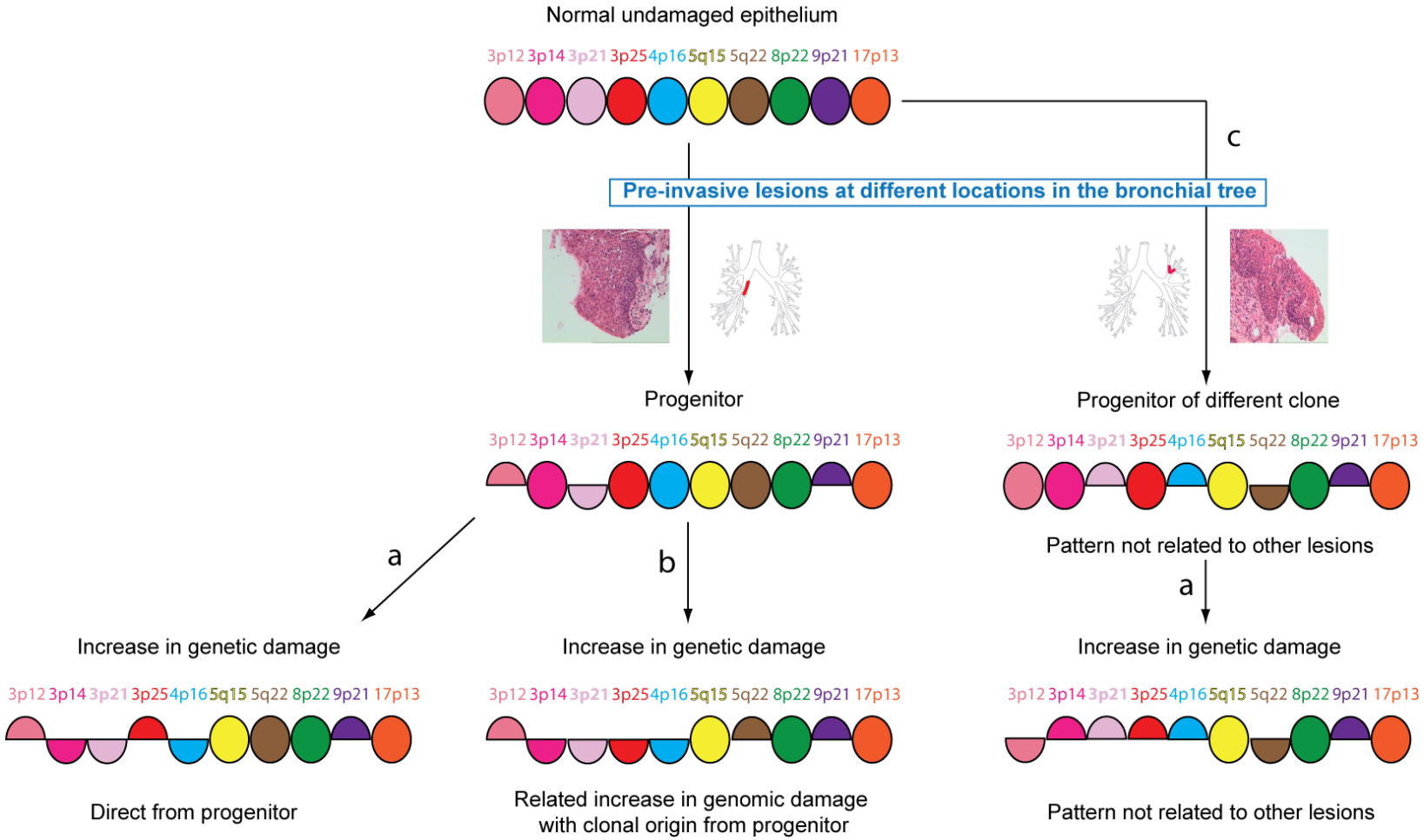


Table 4.7: Genes within the minimally deleted region on 4p16 and their putative actions.

Gene ID	Name	Biochemical action	Biological function
CYTL 1	Cytokine-like 1	Cytokine-like secretory protein Extracellular space receptor binding and signal transduction	Unknown
STK32B	Serine / Threonine kinase 32B	Serine / Threonine protein kinase	Involved in cell communication and signal transduction. Abnormalities implicated in malignant transformation.
EVC2	Ellis van Creveld syndrome 2 (limbin)	Action not known	Implicated in “Ellis-van Creveld” syndrome a developmental disorder
EVC	Ellis van Creveld syndrome	Action not known	Implicated in “Ellis-van Creveld” syndrome a developmental disorder
CRMP1	Collapsin response mediator protein	Downstream effector of tissue growth factor	Inhibition of invasion and metastases in lung adenocarcinoma
WFS1	Wolfram syndrome 1 (wolframin)	Endoglycosidase H-sensitive membrane glycoprotein	Mutations associated with autosomal recessive “Wolfram syndrome” possibly with a role in diabetes
PPP2R2C	Protein phosphatase 2B	Regulatory subunit of a Serine / Threonine phosphatase	Part of Protein Phosphatase 2A complex with tumour suppressor function

Further studies using more closely spaced markers, or an alternative approach such as Single Nucleotide Polymorphism analysis would determine whether there was unrecognised 4p16 LOH in the LLL stump carcinoma and the validity of the relationship between 3p-9p-4p16 LOH and the development of invasive squamous cell carcinoma.

3. Features associated with the regression of pre-invasive lesions to normal epithelium

Within the study 4 lesions in 3 patients were found to have regressed to histologically normal epithelium. Three were HGLs (P8 RLL apical, P6 lingula and P4 LUL) and the other was an LGL (P4 LLL). The extent of the LOH was 4-5/10 loci except for P4 HGL LUL which had 9/10 loci showing LOH.

There was consistent 3p and 9p LOH in the lesions (table 4.6), confirming that these changes occur early in the development of pre-invasive lesions. The HGL at P8 RLL apical showed LOH at 3/4 loci on 3p, and LOH at 9p21 and 17p13. There was no 4p16 LOH. The HGL at P6 lingula showed LOH at only 1/4 loci on 3p, but also 4p16, 5q15 and 9p21. The LGL in P4 had a similar pattern of LOH, with LOH at 3p (3/4 loci), 9p and 17p13 but no 4p16 LOH. This suggests that the combination of LOH at 3p, 9p and 4p16 might be required for lesion progression otherwise the lesion regresses. Alternatively, 3p and 9p LOH may occur as early events in the pathogenesis of pre-invasive lesions, with a further switch within the genome that was not tested in the present study. The findings in the HGL at P4 LUL are more difficult to explain. In this lesion there was LOH at 4/4 loci on 3p, and LOH on 9p21, 17p13 and 4p16. This is a similar allelotype to the HGLs that progressed to invasive squamous cell carcinoma, and supports the theory that changes further to those observed in the present study are required to determine the outcome of individual pre-invasive lesions.

The common feature of all of the lesions that regressed was retention of heterozygosity on 8p22. Within the squamous cell carcinomas and the HGLs that progressed to squamous cell carcinoma, there was LOH at 8p22 in the P11 RLL carcinoma, P11 LLL carcinoma and the P11 LUL HGL. In patient P6 the PCR at 8p22 failed giving no information. The LUL stump

HGL that preceded the squamous cell carcinoma in patient P12 and the squamous cell carcinoma at the LLL stump in patient P11 both retained heterozygosity at 8p22. It is unclear whether 8p22 retention of heterozygosity is related to the regression of pre-invasive lesions. Analysis of more lesions that regressed, with a greater density of markers would be required to establish the relationship between 8p22 retention of heterozygosity and lesion regression.

There may have been another molecular change, not within the scope of the present study, that led the lesion to regression rather than progression to carcinoma. Such changes may include LOH in other areas of the genome not covered in the present study, gene amplification or promoter methylation. Overall it is likely that the combination of changes within some HGLs led to regression rather than progression to squamous cell carcinoma.

4. Lesion Heterogeneity

In serial specimens from some lesions e.g. P8 HGL, P12 HGL LLL stump, P4 HGL LUL, significant differences in the LOH patterns were found. At some loci, LOH appeared to be followed by retention of heterozygosity and then LOH. This could be due to technical reasons such as apparent LOH due to absence of a band on the gel caused by an experimental problem with either the PCR or the gel running process. In the present study, the important results were reproduced experimentally and confirmed using another microsatellite marker from the same chromosomal locus. This minimised the possibility of a false result due to a simple technical error. There are several possible explanations for this variation in the allelotypes of samples from lesions in the present study:

a. The molecular damage that generated the abnormal genotype may continue to progress

Cells from a single progenitor within the lesion accumulates molecular damage, the pattern of which may not be the same as adjacent cells from the same progenitor (figure 4.16). Some of this molecular damage may confer a growth advantage, causing the affected clone of cells to populate that part of the evolving lesion, while other changes may lead to apoptosis or cell death of a particular clone of cells, leaving areas of the evolving lesion open

to population by adjacent cells with a growth advantage. These changes would lead to variations in the LOH pattern in different parts of the same lesion.

b. The location of cells within the lesion may influence their development

The proximity of the cells to their blood supply, or their relationship to the leading growth edge of the lesion or adjacent tissue structures may determine the susceptibility of individual cells to further molecular damage, and consequently the pattern of molecular changes in different parts of the evolving lesion.

c. Biopsy may alter the progress and outcome of the lesion

Biopsy creates a physical defect in the lesion, which may then be repopulated by cells of a different clone, which both restores the integrity of the lesion, but also changes the lesion LOH profile. Each biopsy may have sampled cells from different clones, dependent on which clone had repopulated the defect from biopsies taken at the previous bronchoscopy. From lesions analysed after photodynamic therapy[119,120], there is evidence that the disruption of cells induces a local immunological reaction that causes further tissue damage. The physical disruption of lesions by biopsy has not been previously studied, but it may be postulated that the disruption of cells may cause a similar local immunological reaction that may further influence the behaviour and outcome of the lesion or area of bronchial mucosa.

d. Carcinogen-exposed epithelium develops molecular changes that may lead to the formation and progression of lesions throughout the bronchial tree

Carcinogen-exposed epithelium may develop a unique set of molecular abnormalities related to specific fragile sites within the individual[141,142]. This would result in a similar pattern of allele loss and mutations in cells throughout the bronchial tree. Each of these carcinogen-exposed cells has the potential to evolve further mutations, some of which may be lethal, some of which may drive malignant transformation, but which may not follow the same pattern as adjacent cells (figure 4.16). For example in patient P8 allelotype of the RUL sample was different to the histologically normal sample from the RLL apical segment after regression of the HGL in that location. In the RLL apical segment sample there was LOH at

3p25, 9p21 and 17p13. In the RUL there was LOH at 3p21, 3p25 and 4p16. This is in keeping with previous work showing discordant patterns of allele loss in anatomically remote areas of the bronchial tree[110]. This suggests that the molecular damage induced by cigarette smoke may produce consistent changes in some parts of the genome, and variable changes in others.

e. The complex karyotypes of pre-invasive lesions and squamous cell carcinoma

Squamous cell carcinomas of the bronchus are known to have complex karyotypes, typically polyploid[143]. LOH analysis does not allow for changes in copy number that accompany polyploidy, but show the relationship in terms of PCR product quantity between the 2 alleles amplified during the reaction. LOH is defined as the absence or a reduction in the quantity of one of the alleles. In a polyploid genome, successive loss of alleles may result in LOH, then apparent retention of heterozygosity and then LOH once again.

Carcinogen induces DNA damage at susceptible sites and so chromosomal and DNA abnormalities induced by carcinogens (such as those in cigarette smoke) may induce abnormal segregation of chromosomes in mitosis[90]. This can result in a gain of genetic material in a daughter cell. Apparent retention of heterozygosity after LOH may be due to fortuitous gain of material in this way. In the present study there was more frequently allele imbalance rather than complete loss of an allele detected by LOH analysis which may be explained by these mechanisms. However, whilst feasible in a single cell, this sequence of changes is unlikely to occur in every cell of a lesion. Furthermore, in some samples, complete LOH was found which suggests that this model does not explain all the observations and that there is LOH in some loci that are diploid. Techniques looking at the actual copy number of individual chromosomal loci within lesion cells in the samples under study would clarify this issue.

f. Regression of lesions may occur with new lesions populating the resulting defect in the bronchial epithelium

Genetic changes may confer a growth advantage to a clone of cells adjacent to a pre-invasive lesion. As the pre-invasive lesion undergoes regression this adjacent clone of cells

may expand to replace the pre-invasive lesion. This adjacent clone of cells may carry a different set of genetic changes from the preceding pre-invasive lesion, and so not be programmed to regress.

An alternative explanation is that the entire bronchial epithelium carries genetic changes induced by carcinogen (supported by the finding of genetic loss in histologically normal epithelium in both the present and in previous studies)[102,103] which were detected in the samples after the bronchial epithelium repaired itself following the regression of the pre-invasive lesion. The absence of genetic loss in some samples from bronchoscopically and histologically normal epithelium suggests that the entire epithelium does not always carry carcinogen-induced genetic damage, but it is likely that localised areas of genetic loss occurs, the detection of which is outside the scope of the present study. It is similarly possible that these changes are markers of and related to more important changes elsewhere in the genome that drive the behaviour of pre-invasive lesions.

Further work is required to determine which theory is correct.

5. Histologically normal epithelium

In the location of previous pre-invasive lesions

Pre-invasive lesions were observed to regress to bronchoscopically and histologically normal epithelium in P6 (lingula HGL), P8 (RLL apical HGL) and P4 (LUL HGL and LLL LGL). In P6, the three samples showing histologically normal epithelium taken after regression of the lingula HGL (months 2, 6 and 14) show that they did not evolve from the HGL and were not related to each other. The histologically normal sample at month 24 from the RLL apical segment in P8 showed a different allelotype to the preceding HGL from the same bronchoscopic location. A different allele was lost at 17p13 (D17S 1176) compared to the HGL and there was LOH at 3p25 that had not been present on the HGL. The LOH at 3p14, 3p21 and 5q15 that had been present in the HGL was not found in the histologically normal epithelium. After regression of the HGL, the histologically normal LUL samples from P4 at months 32,43 and 50 showed less extensive genetic loss than the preceding HGL. The

molecular studies confirm that the histologically normal epithelium was not clonally related to the preceding HGL, and the histologically normal samples were not clonally related to each other.

These data suggest that after regression, HGLs are replaced by cells of a different clone. These may originate from intact bronchial epithelium adjacent to the HGL, or alternatively, basal cells from the edges of the area of the previous HGL may have populated the defect in the epithelium left by the HGL or perhaps biopsy debulking the HGL. The clone of cells repopulating the epithelium may carry molecular abnormalities of differing extent and pattern. The extent of these abnormalities are typically less than the preceding HGL but greater than histologically normal epithelium from bronchoscopically normal locations in which there had not been a previous pre-invasive lesion. The pattern of molecular abnormalities in such epithelium may suggest an origin from a common precursor, or be unrelated to any previous identified abnormality within the subject.

Histologically normal epithelium with no previous abnormality

In patient P12 there was no histological evidence of resection margin carcinoma or HGL when the original carcinoma was resected. Two years later a HGL was found at the LUL stump. The allelotypes of the original carcinoma and the LUL stump HGL were similar, but the lack of 5q22 LOH (at D5S 644) in the LUL stump HGL and the different alleles lost at 9p21 (D9S 162) suggests that the LUL stump HGL was formed from a different clone of cells to the original LUL carcinoma although both clones shared a common progenitor cell. The LUL stump HGL was not a recurrence or metastasis of the original LUL carcinoma but a new abnormality. There are three possible explanations for the finding of a different clone of cells to the original carcinoma at the LUL stump:

a. The progenitor cell for the clone that generated the HGL may have been present at the resection margin, but not identifiable using standard histological techniques. This may have subsequently evolved to form the HGL clone of cells.

b. The LUL stump HGL clone of cells may have been present at the resection margin at the time of the resection but had not formed a lesion at that stage. These cells may have subsequently proliferated to generate the HGL.
c. The HGL may have evolved directly from the cigarette-smoke exposed epithelium at the resection margin.

Using current techniques it is not possible to verify which of these theories is correct.

Samples were taken 33 months apart from histologically and bronchoscopically normal epithelium of the LLL in P12. The cells within the samples carried molecular abnormalities, the allelotypes of which suggested that they had the same progenitor as each other, and also the LUL stump HGL and the LMB HGL, but had sufficient differences to be from different clones of cells. There are 2 possible explanations:

a. It is possible that the original clone of cells at the LLL orifice at month 0 regressed and a new clone of cells had populated that area of mucosa by month 33.
b. The process of biopsy may have removed the clone of cells entirely, and a new clone of cells had entered that part of the mucosa as part of the healing process.

There was no bronchoscopic or histological abnormality in the lingula in patient P4. No genetic loss was detected in the specimen from this location at month 50. At month 57 LOH was found at 3p12, 3p14 and 17p. No bronchoscopic or histological abnormality has been observed in the lingula during the year since that sample was obtained. New genetic loss was found up to 4 years after smoking cessation in this previously bronchoscopically, histologically and genetically normal bronchial epithelium. The mechanism by which genetic loss occurs after smoking cessation is not known. There may be carcinogen-induced susceptibility without actual genetic loss within the bronchial epithelium in which a later event may then result in detectable genetic loss in susceptible loci of the genome.

Molecular abnormalities have been found using LOH analysis[102,103,108] and CGH[71] in the bronchial epithelium of smokers without bronchoscopically or histologically visible lesions. It is unclear which abnormalities are markers of cigarette smoke exposure and which drive the

development of pre-invasive lesions and squamous cell carcinoma. It has been suggested that molecular changes within bronchial epithelial cells precede and then drive the development of pre-invasive lesions and that over time a HGL or squamous cell carcinoma might develop. The timescale over which lesions develop from cigarette smoke exposed epithelium is not known, but the data show that cells may carry molecular abnormalities without necessarily forming a lesion for at least 4 years and that 4 years after smoking cessation, new molecular abnormalities may develop in histologically normal epithelium.

6. Implications for the interpretation of lesion natural history data

It is currently thought that a pre-invasive lesion at a bronchoscopic location is the same lesion when sampled at different time-points. This has informed the conclusions of all the clinical studies of the natural history of pre-invasive lesions[26,27]. The lesions were sampled at a number of time-points with intervals ranging from 4 months to a year depending on the histopathological features of the lesions under study. The molecular studies presented have shown that a lesion of a given histology at a single bronchoscopic location may not be the same lesion genetically when sampled serially over a period of months. The evidence suggests that the lesion was unlikely to have developed from the previous lesion at that location, but from an earlier progenitor cell. This suggests that the original lesion may have regressed, and been replaced by cells of a different clone from adjacent epithelium or by cells from the original progenitor stem cell direct. This original lesion may have regressed, been removed by biopsy, or disrupted by biopsy and subsequently undergone regression.

Due to the low numbers of lesions in the molecular studies, the incidence of this phenomenon could not be estimated. If this is a common occurrence, then this would confound longitudinal studies of pre-invasive lesions, as the natural history of a given lesion cannot be evaluated from clinical observation alone. This highlights the need for combined molecular and clinical studies to evaluate in detail individual areas of the bronchial tree, to ascertain the true natural history of the lesions under study. Of course, as previously described, such studies are inevitably affected by the biopsy and therefore disruption of

lesions, but until non-destructive sampling techniques are developed that are as reliable as biopsy and histopathology, this will remain the investigative method of choice.

7. Summary and future directions

Previous studies have noted allele-specific mutations within the genome of bronchial epithelial cells in remote parts of the bronchial tree[94,109,111]. This suggests that in some situations, carcinogen causes consistent effects on the genome of bronchial epithelial cells. In the present study, lesions in remote parts of the bronchial tree carried similar mutation patterns, with the same alleles lost at the majority of the markers used. However, in many of the lesions, significant differences in the allelotypes were found. These data are in agreement with the findings of Boyle and colleagues[110], who found both "concordant" and "discordant" mutations when they compared pre-invasive lesions and synchronous carcinomas in specimens resected for lung cancer. There were "discordant" patterns of allele loss in 2 out of 8 patients in their study, which means that different alleles were lost at some chromosomal loci in the pre-invasive lesions and the carcinoma. The remainder of the patients showed loss of the same allele at each chromosomal locus that showed genetic loss within both the pre-invasive lesions in the bronchial tree and their synchronous carcinomas ("concordant" mutations).

These data suggest that on a background of susceptibility of parts of the genome to the mutational effects of carcinogen, genetic damage may occur to other parts of the genome that is not consistent throughout the airways. This may be the cause of the differences in the natural history of individual lesions. Whereas some of the changes, such as 3p LOH and 9p LOH are thought to occur early in pre-invasive lesion development, others occur later such as 17p13 and 5q22 LOH. The consistent early changes may be a marker of smoking, rather than part of the process by which malignant disease develops[102]. The present study, along with previous studies has shown that 3p LOH increases in extent as the lesion progresses from normal epithelium to squamous cell carcinoma[104,109,111,112]. This suggests that the extent of LOH on 3p may be related to the development of malignancy within an individual lesion.

Some molecular changes are probably markers of smoking-related damage, while others are related directly to the development of malignancy, changes that occur at the transition from pre-invasive lesion (carcinoma-in-situ) to invasive disease. It is known that this represents a significant biological shift in the nature and behaviour of the lesion, as the survival with radical treatment falls from 90% at 5 years for carcinoma-in-situ[9] to 60% for stage 1 micro-invasive carcinoma[144]. This is largely due to distant spread, which suggests that the lesion has acquired metastatic potential at the point of invasion. These phenotypic changes are underpinned by molecular changes, which are the result of carcinogen exposure.

In the present studies, LOH at the majority of 3p, with LOH at 9p, both changes associated with early stages of pre-invasive lesion development, occurred in concert with LOH at 4p16, a locus with a number of candidate tumour suppressor genes, and genes potentially responsible for the development of invasion. This suggests that studies of lesions at this transition point should yield consistent profiles that may define the lesions at highest risk of malignant transformation.

Over the past 4-5 years, attention has focused on gene amplification and overexpression of oncogenes as a mechanism of lung carcinogenesis. In a study of high-grade pre-invasive lesions identified by autofluorescence bronchoscopy, the copy number of *TP63, MYC, CEP6 and CEP3* increased with increasing lesion histological grade[145]. When 1-2 markers were amplified, the risk ratio of lung cancer development was 4.23, but when 3-4 markers were amplified the relative risk rose to 11. In particular, overexpression of *TP63* (3q28) and *MYC* (8q24) were associated with an increased risk of malignant change in the lesion. From these data, attention has focused on 3q amplification as an important driver of squamous cell carcinoma development. In a series of studies, McCaughan and colleagues have shown that HGLs have amplification of 3q that is not present in LGLs[146]. In HGLs progressing to squamous cell carcinoma there was incremental amplification of 3q, with the oncogene *SOX2* showing consistent amplification at the transition from carcinoma-in-situ to invasive squamous cell carcinoma[147].

These findings suggest that the molecular basis of carcinogenesis is multifactorial, with consistent areas of genetic loss and amplification generating the malignant phenotype. Previous studies were limited by the small quantities of genetic material available from each lesion for study, but newer techniques have made higher resolution and genome-wide studies of these specimens feasible. Further studies would explore and expand these findings by obtaining more detailed allelotypes and combine genetic loss with amplification and gain of genetic material to generate a fingerprint that might be able to predict the transformation of an individual lesion to invasive malignant disease. Treatment at this early stage might reduce the mortality from squamous cell carcinoma of the bronchus by allowing targeted therapy to the lesions at highest risk. In addition, this would allow the targeting of invasive investigations to those patients carrying high risk lesions.

Future directions:

1. A re-evaluation of the natural history of pre-invasive lesion studies. This would involve serial samples of lesions backed up by molecular profiles using a genome-wide approach such as CGH or SNP analysis. This would determine whether the lesion under observation remains the same lesion during follow-up.
2. The relationship between a lesion and the surrounding bronchial mucosa. It would be necessary to establish the bronchoscopic, histologic and molecular extent of lesions to determine accuracy of each technique in the evaluation of bronchial pre-invasive lesions.
3. The entire molecular set of abnormalities in an individual lesion, including areas of gain and loss, coupled with RNA or immunohistochemical techniques to evaluate the effect of these changes on the gene products downstream and therefore cell physiology. This would include CpG island Methylation status, and be coupled with analysis of the histological and bronchoscopic progress of the lesions studied, to provide an accurate evaluation of the bronchial mucosa. This would also provide detailed analysis of the molecular switches involved in the invasion and metastasis of lesions.

4. Serial sampling of individual lesions with a non-disruptive technique, maintaining lesion integrity and not altering the number of cells. This would allow an unbiased evaluation of lesions in vivo.

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Glossary of Abbreviations

AFL	Autofluorescence bronchoscopy
ASD	Angiogenic squamous dysplasia
ChT	Chemotherapy
CI	Confidence intervals
CIS	Carcinoma-in-situ
COPD	Chronic obstructive pulmonary disease
CP	Chemoprevention
CRP	C-reactive protein
HGL	High-grade lesion
LGL	Low-grade lesion
LIFE	Light-induced fluorescence endoscopy
LLL	Left lower lobe
LOH	Loss of heterozygosity
LUL	Left upper lobe
MiD	Mild dysplasia
MoD	Moderate dysplasia
MoD+	Moderate dysplasia, severe dysplasia, carcinoma-in-situ and squamous cell carcinoma combined
MPA	Metaplasia
NAD	No abnormality detected
PDT	Photodynamic therapy
RLL	Right lower lobe
RML	Right middle lobe
RT	Radiotherapy
RUL	Right upper lobe
SCLC	Small cell lung cancer
SD	Severe dysplasia
Sens	Sensitivity
SQC	Squamous cell carcinoma
WLB	White-light bronchoscopy

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